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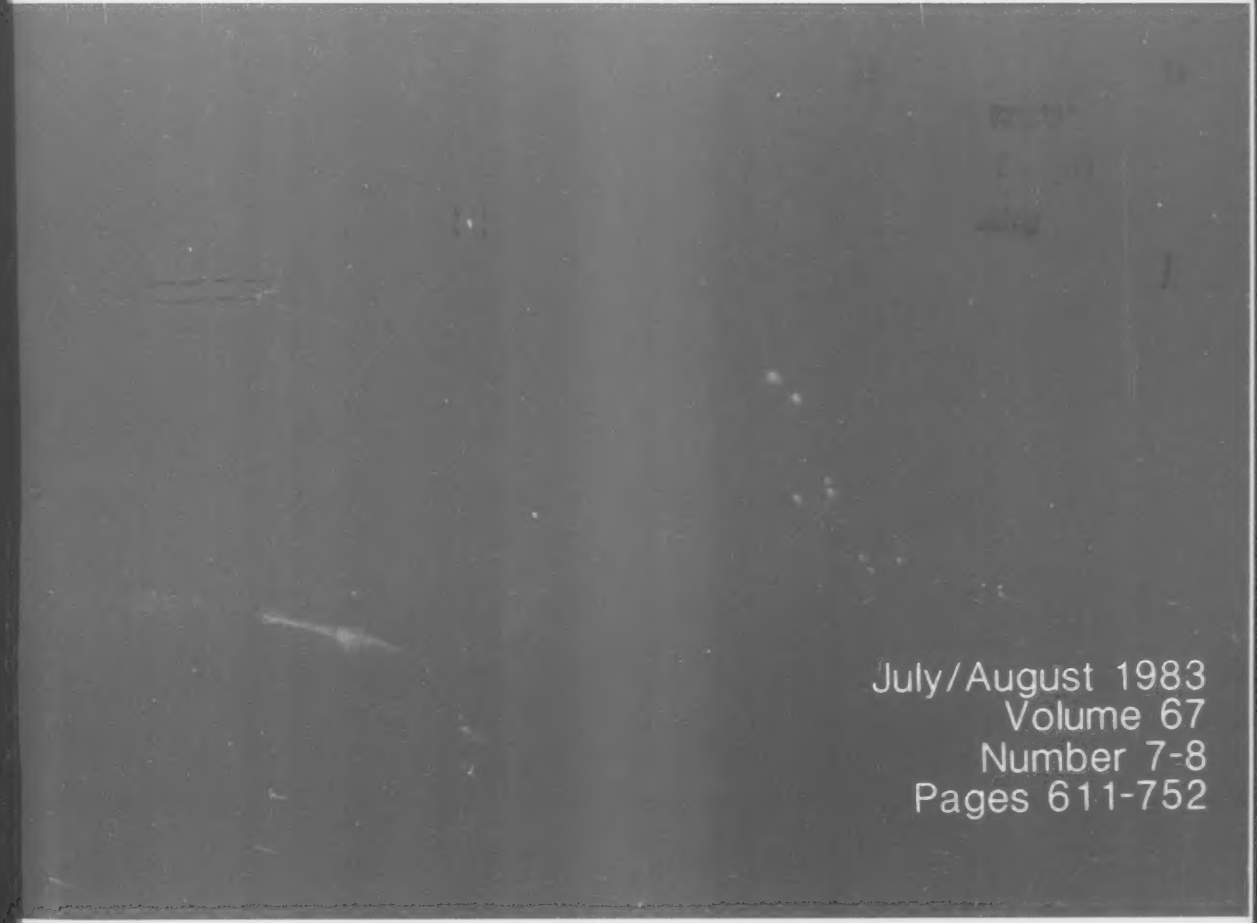

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Leukocyte Recovery With Short-Chain RNA Fragments in Cyclophosphamide-Treated Rabbits[1]

Mirko Beljanski,* Michel Plawecki, Pierre Bourgarel, and Monique Beljanski[2]

Single-stranded short-chain RNA fragments, obtained by mild degradation of purified *Escherichia coli* ribosomal RNA(s) with pancreatic RNase A, exhibit particular biologic activities in vitro and in vivo. In vitro, these RNA fragments are used by DNA-dependent DNA polymerase I as primers to initiate the replication of DNA(s) isolated from rabbit bone marrow and spleen; they are inactive with DNA isolated from several normal tissues and cancerous cells. Administered iv, RNA fragments restore a normal level of circulating leukocytes in rabbits treated with high doses of cyclophosphamide (CP). Granulocyte/lymphocyte balance, upset by daily CP administration, is also restored during the increase of both types of cells. No toxicity is observed, and numerous repeated doses of RNA fragments show no cumulative effect and do not lead to loss of leukopoietic stimulating activity. Tumor-bearing mice can be protected by RNA fragments against the toxic effect of CP without impeding the anticancer activity of this drug. [Cancer Treat Rep 67:611–619, 1983]

The primary step of cell division requires the replication of DNA by DNA-dependent DNA polymerase. As shown experimentally in different biologic systems, this enzyme uses special, naturally produced short-chain RNA(s) (1–4) as primers to initiate the new DNA chain.

In previous articles (5–7) we have shown that specific RNA fragments obtained from the product of enzymatic breakdown of bacterial ribosomal RNA(s) act as primers for in vitro replication of DNA(s) originating from various sources. The RNA fragments obtained by this method are single-stranded, and their nucleotide composition appears to determine their specificity of action.

Among these RNA primers, we have used those which specifically act as primers in the in vitro replication of DNA isolated from blood cell-forming tissues: bone marrow and spleen. These RNA fragments are not transcribed into DNA(s), and once their in vivo action terminates they are removed from the DNA template and degraded.

Many drugs, most cancer chemotherapeutic agents, and ionizing radiation have toxic secondary effects on the hematopoietic system. In particular, they impair the regenerative capacity of the bone marrow, inducing leukopenia and thrombocytopenia and thus increasing infection risks. Moreover, these effects require suspension of chemotherapy, during which time cancer cells may multiply. Using active RNA fragments, it is possible to slow or arrest, in vivo, the secondary toxic effect of an anticancer drug.

In this paper, we describe the production method for RNA fragments active on leukopoiesis. These RNA fragments have been characterized, and their in vitro and in vivo activities are reported here. Preliminary results concerning the action of these RNA fragments on thrombopoiesis and leukopoiesis have been presented (8,9).

MATERIALS AND METHODS

Chemical and biologic products.—Phenol (grade A) and chloroform were obtained from Prolabo, Paris, France, and sodium lauryl sulfate was obtained from Serlabo, Paris, France. Pancreatic RNase (4× crystallized) and DNase (grade A) were supplied by Worthington, Freehold, NJ. Poly AG, Poly A, ^{3}H-Poly A (specific activity, 93 μCi/μmol), and ^{3}H-Poly C (specific activity, 54 μCi/μmol) were from Miles Laboratories, Elkhart, IN. Cyclophosphamide (CP; Endoxan) was from the Laboratoires Lucien, Colombes, France; ^{3}H-uracil (44.5 Ci/mmol) and ^{3}H-guanine (7.5 Ci/mmol) were from the Commissariat à l'Energie Atomique, Saclay, France.

[1]Received July 27, 1982; revised Mar 28, 1983; accepted Apr 29, 1983.

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Animals.—Male New Zealand White rabbits weighing approximately 3 kg were from Charles River Laboratories, France.

Bacterial growth conditions.—A nonpathogenic strain of *Escherichia coli* (K 12) was used. Cells were grown aerobically in a synthetic medium at 36°C (10) and harvested during the exponential growth phase. When radioactive RNA(s) was required, cells were labeled for 6 hrs during the exponential phase by the addition of 500 μCi of ^{3}H-guanine and 500 μCi of ^{3}H-uracil/L of synthetic medium.

Isolation of ribosomes and ribosomal RNA(s).—Ribosomes were prepared from cells as previously described (11) and deproteinized by several treatments with phenol and chloroform to isolate r-RNA(s). r-RNA(s) was dissolved in 0.01 M Na acetate and 0.1 M NaCl (pH 5.0), and the solution was centrifuged for 30 mins at 40,000 × *g* to remove most polysaccharide contaminants (12). The supernatant solution was dialyzed against 0.1 M KCl for 18 hrs at 4°C and then against distilled water for 1 hr at 4°C. RNA yield was determined by measurement at 260 nm, and integrity of r-RNA(s) was checked by polyacrylamide gel electrophoresis (11).

Degradation of ribosomal RNA(s) by pancreatic RNase A.—Pancreatic RNase A (15 mg) was added to a solution of RNA (1 g) in distilled water (50 ml) and the mixture was incubated at 36°C for 20 mins. The reaction was stopped by adding 1 vol of phenol containing 10% distilled water; the mixture was stirred at room temperature for 10 mins and centrifuged at 12,000 × *g* for 10 mins to separate phenolic and aqueous phases. The aqueous phase was removed and treated again as before with 1 vol of 10% aqueous phenol. The second aqueous phase was then shaken for 10 mins with 1 vol of chloroform and after centrifugation the aqueous phase was recovered. This step was repeated three times, and the final aqueous phase was dialyzed against 2.5 L of distilled water for 16 hrs at 4°C under axenic conditions. The amount of nondialyzable RNA was determined by measurement at 260 nm (yield, 50%–60%), the dialyzed solution was freeze-dried, and the residue was stored in a dry environment. The products that stimulate leukopoiesis and platelet formation are termed Beljanski leukocyte restorers (BLR).

Analytic techniques.—Proteins were measured by the method of Lowry et al (13), and DNA was measured by the diphenylamine reaction (14). RNA base ratios were determined as follows: 150 μg of BLR was hydrolyzed at 100°C in 1 N HCl for 1 hr (boiling water bath), the hydrolysate was evaporated to dryness in a dessicator, and the residue was dissolved in 0.02 ml of distilled water. Samples of the solution were analyzed by chromatography on Ecteola cellulose plates according to the method described by Björk and Svensson (15). UV-absorbing spots were located (purine bases and pyrimidine nucleotides), cut out, and eluted with 0.1 N HCl. The concentrations of eluted compounds were determined using the following absorption maxima coefficients: A, 13; G, 12.8; C, 11; and U, 10 (16).

Isolation of DNA and DNA polymerase.—DNA was isolated from gently broken tissues by phenol/chloroform extraction in the presence of 2 M $NaClO_4$ and 0.01 M EDTA (17). *E. coli* DNA-dependent DNA polymerase I (EC 2.7.7.7) was purified from bacterial extracts as previously described (18).

Determination of leukocyte count in circulating blood of rabbits.—Blood was taken from the marginal vein of the ear using complexion as an anticoagulant, and leukocyte counts were determined using a Coulter counter model ZBI. For differential leukocyte counts the May-Grünwald-Giemsa staining method was used, and 200–300 leukocytes were counted under the microscope. Leukocytes were classified as granulocytes (neutrophils, eosinophils, and basophils) and lymphocytes, including monocytes.

Incubation medium for in vitro DNA replication.—The medium contained (per 0.15 ml): Tris-HCl buffer (pH 7.65), 25 μmols; $MgCl_2$, 2 μmols; 4 dXTP, 5 nmols each (+ ^{3}H-TTP, 50,000 counts/min [cpm]); DNA, 0.2 μg; BLR when indicated, 4 μg; and DNA-dependent DNA polymerase, 80 μg. Incubation was for 10, 20, and 30 mins at 36°C. Trichloroacetic acid (5%)-precipitable material was filtered on a Whatman GF/C glass filter, washed, and dried, and radioactivity was measured in a Packard liquid scintillation counter.

In vitro degradation of ^{3}H-BLR by blood plasma from healthy rabbits.—The incubation mixture (final volume, 0.15 ml) contained: 25 μmols of Tris-HCl buffer, pH 7.65; 200 μg of ^{3}H-BLR (7000 cpm); and 0.01 ml of plasma (0.7 mg of protein). A parallel experiment was performed with ^{3}H-Poly A (200 μg, 7200 cpm) and ^{3}H-Poly C (200 μg, 7160 cpm). Incubation was at 36°C. To stop the reaction, alcohol (95°) was added in excess, and 5 μmols of KCl was added to precipitate the ^{3}H-labeled insoluble material. The precipitate was filtered on a Whatman GF/C glass filter, washed with alcohol, and dried; radioactivity was measured in a Packard liquid scintillation counter.

RESULTS

Biochemical Characteristics of BLR

Chemical and physical characterization of BLR shows that they are single-stranded RNA fragments (not hyperchromic), practically devoid of DNA and proteins: ratio (260/280 nm), 2.23 ± 0.05; quantity (mg) by UV (260 nm), 1.98 ± 0.05; % protein(s) [method of Lowry et al (13)], 0.27 ± 0.08; and % DNA-like material [diphenylamine reagent (14)], 0.29 ± 0.08. For the different analyses, 2 mg of each BLR sample was weighed. Results are mean values from four different samples ± 1 SE.

TABLE 1.—Base ratio (G + A/C + U) of r-RNA and BLR obtained by degradation of the same r-RNA*

Base	r-RNA (23 S + 16 S)	BLR
G	27.90 ± 0.30	41.25 ± 0.70
A	24.80 ± 0.20	29.30 ± 0.50
C	24.10 ± 0.10	16.10 ± 0.40
U	23.20 ± 0.10	13.85 ± 0.35
G + A/C + U	1.11 ± 0.01	2.35 ± 0.03

* Values = mols/100 mols of analyzed nucleotides. r-RNA and BLR were hydrolyzed and analyzed as described in the Materials and Methods section. For extension coefficients, *see* ref 16. Results are mean values from 4 different samples ± 1 SE.

Base ratio analysis shows that BLR contain an excess of purine over pyrimidine bases (G + A/C + U = 2.35), while in intact r-RNA this ratio is about 1.10 (table 1). The average electrophoretic mobility of BLR is the same as that of Poly A and has a sedimentation coefficient of 3 S (fig 1). This suggests that these RNA fragments contain an average of about 40 nucleotides, which is within the size range of DNA replication primers (19). Their priming action on DNA replication in vitro was tested using bone marrow and spleen DNA(s). Choice of this DNA(s) was guided by the observation that injected radioactive BLR are found in relatively high amounts in

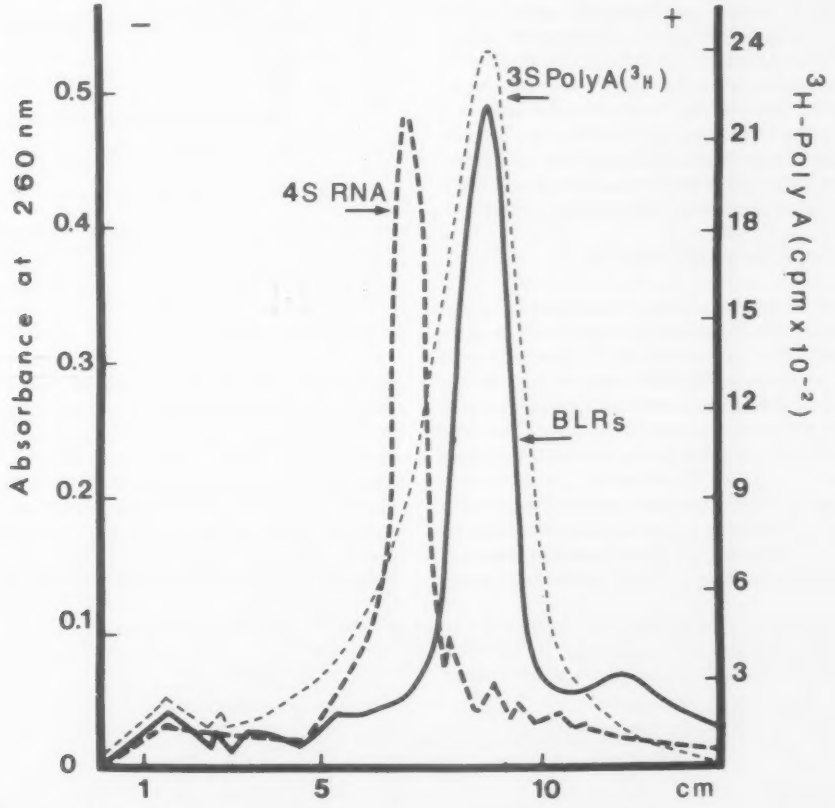

FIGURE 1.—Polyacrylamide gel electrophoresis of BLR. 4 μg of BLR was mixed with ³H-Poly A (3 S; 14,000 cpm), used as a marker, and submitted to electrophoresis on polyacrylamide gel (5% wt/vol) for 90 mins at 4 °C with 5 mA/tube. 4 S RNA was separately run as a marker. The position of UV-absorbing material 4 S RNA and BLR was detected by densitometer tracing in a Beckman spectrophotometer at 260 nm. The position of ³H-Poly A was determined by measuring the radioactivity in 1-mm thin slices of polyacrylamide gel using a Packard liquid scintillation counter.

the bone marrow and spleen (20). It was furthermore essential to ascertain whether BLR act differently on DNA of normal and cancerous cells. When DNA-dependent DNA polymerase I was incubated in a complete system containing a labeled deoxyribonucleoside-5′-triphosphate but in the absence of exogenous RNA primer, it could synthesize a very limited amount of DNA in a reaction which soon reached a plateau. When BLR were added to such a system using bone marrow or spleen DNA as a template, replication was highly stimulated (fig 2), whereas when brain or kidney DNA was used, no noticeable stimulation was observed. These results suggested that BLR could act in vivo as DNA replication primers in the bone marrow and spleen, where they are retained.

We then sought to find out whether BLR exerted any stimulating action on the in vitro replication of cancer DNA. To this effect, they were tested using DNA(s) isolated from various healthy and cancerous tissues. When DNA(s) from healthy lung tissue was used as a template in the complete system, we observed only a slight stimulation of DNA replication. On the other hand, when template DNA(s) prepared from cancerous lung tissue was used, the replication was slightly decelerated by the presence of BLR (table 2). The same variations were shown with other DNA prepared from healthy and cancerous tissues. These results indicated that BLR would not stimulate cancer DNA replication in vivo and would not result in malignant cell multiplication in animals.

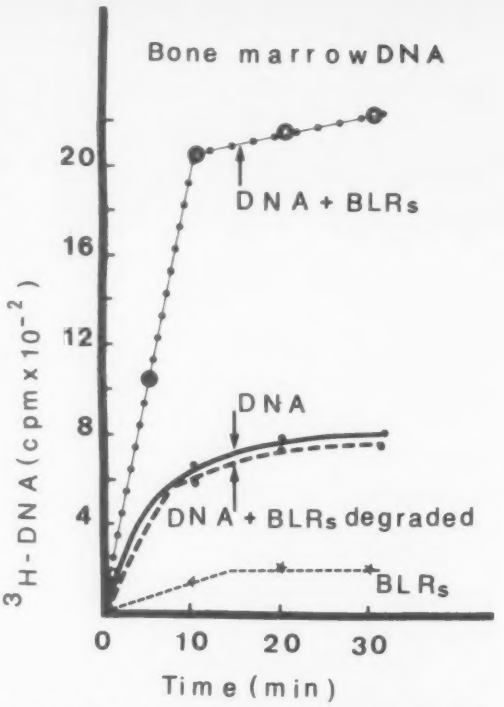

FIGURE 2.—BLR: in vitro primers for bone marrow DNA replication. Solid circles = ^{3}H-DNA synthesis with a complete incubation mixture. Open circles = stimulation of ^{3}H-DNA synthesis in the presence of 4 μg of BLR added to the incubation medium at 0 time. ---- = degraded BLR used; no stimulation occurs. ×--× = bone marrow DNA omitted from medium; 4 μg of BLR added.

Degradation of BLR by Rabbit Plasma

To evaluate the extent of BLR degradation as a function of time, plasma from healthy rabbits was prepared and incubated with ^{3}H-BLR at 36°C. Parallel experiments were performed with ^{3}H-Poly C or Poly A. The half-life of ^{3}H-BLR was 8 mins and that of Poly C, 1 min, while Poly A was poorly degraded (fig 3). BLR, which contain an excess of purine bases, showed a relatively high resistance to plasma RNase action. The activity of plasma RNase was also tested after rabbits had received BLR iv. Blood plasma was prepared just before injection and every hr thereafter for 7 hrs. Table 3 shows that after BLR injection, plasma RNase exhibited a higher degradation activity toward ^{3}H-BLR than that found in the plasma before BLR administration. Increased RNase activity was manifest for 5-6 hrs and then returned to normal value. These observations did not tell us if injected BLR had induced the increase in the amount of plasma RNase or if some RNase-activating factor(s) had appeared in the presence of BLR. The period of increased plasma RNase activity corresponded to transient fever response induced by BLR injection in rabbits.

TABLE 2.—Effect of different BLR doses on replication of DNA purified from human lung: healthy and cancerous tissues*

BLR dose (μg)	Healthy lung DNA (0.1 μg)	Cancer lung DNA (0.5 μg)
0	1000 ± 80	8000 ± 350
4	1850 ± 150	7850 ± 210
8	1750 ± 120	7800 ± 250
12	1400 ± 100	7300 ± 180

* Values = cpm. Incubation conditions are as described in the Materials and Methods section. Results are mean values from 3 different experiments ± 1 SE. Time of incubation: 10 mins.

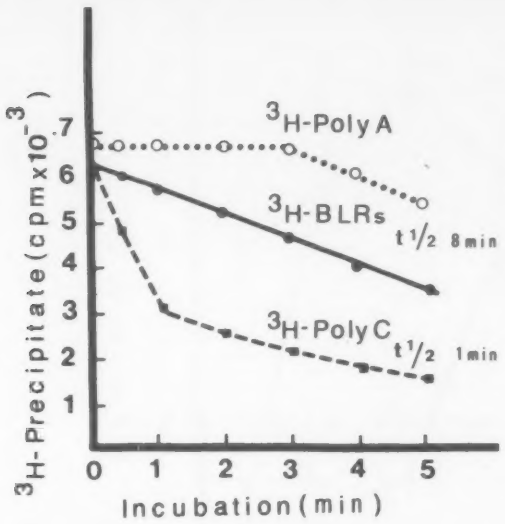

FIGURE 3.—In vitro degradation of ^{3}H-BLR by plasma of healthy rabbits. Degradation of BLR, which contain 2.3-fold more purine than pyrimidine bases, was compared with that of two homopolymers: Poly A and Poly C. Results are expressed as cpm. Half-life (0.693/K) was calculated using the values in the linear portion of the curves and represents mean value for 3 different experiments.

BLR Activity and Integrity

Phosphatase activity of RNase A used to prepare BLR removed 3′-phosphate from RNA fragments, thus providing the 3′-OH group necessary for priming activity. The same reaction took place in the bone marrow of animals.

After incubation with *E. coli* alkaline phosphatase, BLR retained their normal leukopoietic activity. Treatment with RNase A and T_1 strongly reduced the activity of BLR in vitro (table 4).

If degraded by alkali, BLR lost both in vitro and in vivo activities (table 5).

TABLE 3.—Plasma RNase activity after BLR injection*

Time after injection (hrs)	RNase activity (cpm)
0	10,000 ± 150
1	7541 ± 160
2	6122 ± 73
3	6159 ± 42
4	6143 ± 30
5	6304 ± 36
6	7316 ± 110
7	9870 ± 135

* At 0 time, 1 mg/kg of BLR was injected iv; blood samples were collected just prior to BLR injection and every hr thereafter. Each result represents the mean value from 3 experiments ± 1 SE. $P < 0.001$ when compared to values obtained at 0 time.

TABLE 4.—Effect of RNases or DNase on BLR activity and in vitro DNA synthesis*

Additions to complete incubation mixture	Spleen DNA synthesis (cpm)
None (mixture alone)	536 ± 15
BLR (4 μg)	1877 ± 36
BLR (4 μg), RNase A (2 μg), and RNase T_1 (1 μg)	641 ± 21
BLR (4 μg) and DNase (2 μg)	132 ± 10

* For incubation conditions, *see* Materials and Methods section. Time of incubation: 10 mins. Results are mean values from 3 different experiments ± SE.

Leukopoietic Activity of BLR in CP-Treated Rabbits

CP, an alkylating agent used for human cancer therapy, has, like almost all the antimitotic drugs, a secondary toxic effect on the production of wbcs.

New Zealand White rabbits were given 23–35 mg/kg of CP iv daily. These high CP doses induced a progressive decrease in the circulating leukocyte count, which fell from 10,000/mm³ (mean value) to 3500–5000/mm³ on Day 8 (about one-half the normal leukocyte count). If rabbits did not receive BLR, they died on Day 13 ± 3 (ten rabbits). If, when the leukocyte count was lowest (3500–5000/mm³), rabbits were given an iv injection of 1 mg/kg of BLR in sterile physiologic saline solution, the leukocyte count increased and reached a normal value 48 hrs after BLR injection (fig 4). The wbc count remained at top normal level for a few days, then decreased because of the daily CP administration. The mean increase in leukocyte count was 154% ± 11% (P . 0.01 by standard Student's t test). BLR-induced changes in leukocyte number lasted about 4–6 days. Then another BLR injection was necessary to ensure survival of rabbits. BLR therapy could be repeated every seventh day for 2 or 3 mos.

Increased leukocyte number obtained with BLR did not exceed 300% of the lowered count in CP-treated rabbits, nor 100% of the normal leukocyte value in untreated rabbits. As may be seen from table 5, an excess dose of BLR

TABLE 5.—Effect of different BLR doses on circulating leukocyte counts of CP-treated rabbits

BLR dose (mg/kg)	% increase from control*
0.3	160 ± 12
1	154 ± 11
3	187 ± 15
1, pretreated with alkali	21 ± 3

* % leukocyte increase 48 hrs following iv BLR administration, compared with the leukocyte count at 0 time (mean value, 4820 ± 210) in CP-treated rabbits. Results are mean values of 6–10 rabbits ± 1 SE.

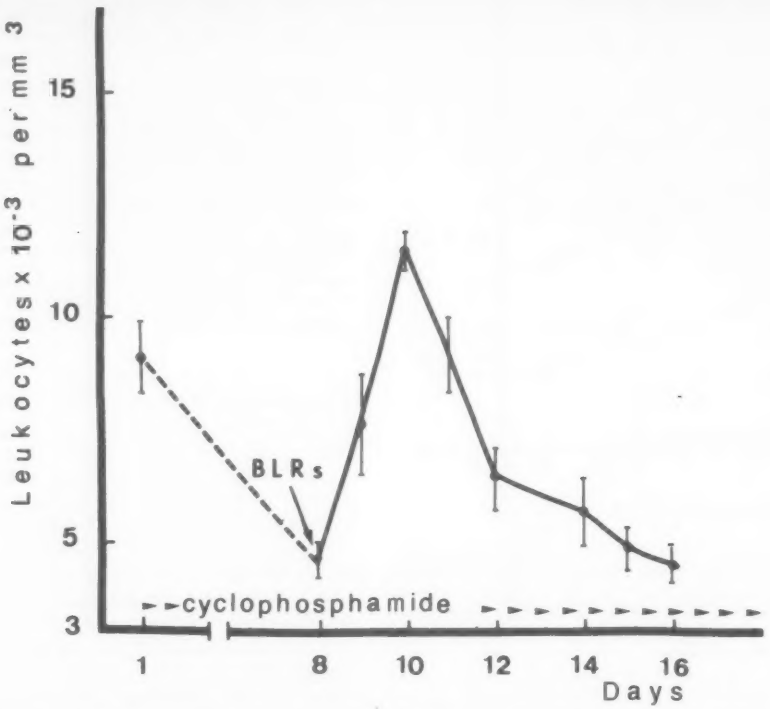

FIGURE 4.—Duration of leukocyte increase induced by BLR. Rabbits were given daily 23–25-mg/kg iv injections of CP starting on Day 1. Leukocyte count was determined every day. When it had fallen below one-half the initial value, the rabbits received 1 mg/kg of BLR iv. The curve represents the mean value for 10 rabbits ± SE.

gave the same response as 0.3 mg/kg. This suggests that a very powerful cellular regulation process is involved in leukocytogenesis and that BLR action is of a physiologic type. In fact, numerous repeated BLR injections led neither to a cumulative response nor to loss of the leukopoietic stimulating activity, and there were no toxic effects or appearance of a tolerance phenomenon (21). In addition, no malaise or pain was observed after BLR injection. No modification of behavior was observed in rabbits given iv BLR doses up to 20 mg/kg. Moreover, no adverse reaction was noted in 20-g (BALB/c) mice injected ip with 0.1–1 g/kg of BLR. Seventy rabbits receiving high daily doses of CP for 30–90 consecutive days and BLR at regular intervals (1 mg/kg/wk) survived in good condition over 1 yr after the CP + BLR treatment was stopped.

Daily use of CP entailed a progressive decrease in the erythrocyte count which became dangerously low after 3 mos. This meant that we had to end treatment when the number of rbcs had fallen below $3 \times 10^6/mm^3$.

The synthetic polyribonucleotides Poly G, Poly A, or Poly AG, injected iv into CP-treated or normal rabbits, failed to stimulate leukocyte formation.

Restoration of Balance Between Granulocytes and Lymphocytes by BLR

Daily CP injection in animals decreased not only the number of circulating lymphocytes but even more strongly that of granulocytes, which decreased to 20% after Day 8. One single iv injection of BLR (fig 5) restored a normal granulocyte/lymphocyte balance, followed by an increase in both types of cells. The CP-induced imbalance reappeared 4–5 days later and could be corrected again by a second BLR injection. Poly AG or Poly A, as well as several other compounds, could not restore granulocyte/lymphocyte balance. On the basis of these results BLR can be considered as naturally occurring substances, which in some instances are perhaps synthesized and used by living organisms to maintain and restore leukocyte level after drug damage.

BLR Treatment and Malignant Cell Development in Mice Undergoing Chemotherapy

The fact that BLR did not stimulate the in vitro replication of DNA from cancerous tissues suggested that they

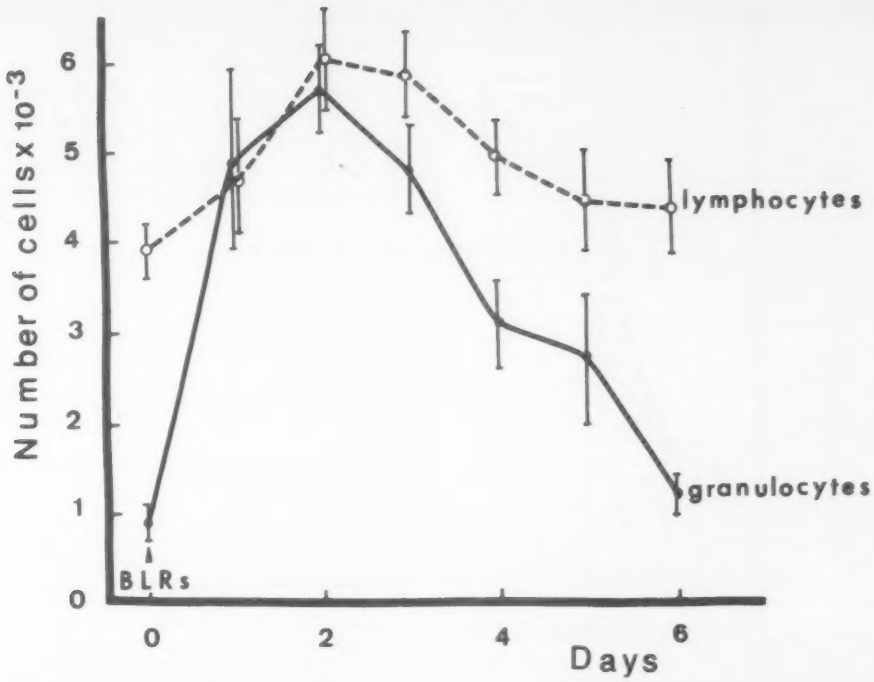

FIGURE 5.—Restoration of balance between granulocytes and lymphocytes by BLR. Conditions are the same as for fig 4. Points = mean values for 10 rabbits ± 1 SE.

would not stimulate malignant cell multiplication in vivo and would not interfere with antimitotic drug activity. To demonstrate this, male Swiss mice inoculated ip with Ehrlich ascitic tumor cells were treated with CP alone or with BLR injections. Weight and survival of the animals were noted during and after treatment (fig 6). All mice from the control group receiving physiologic saline solution died within 3 wks after malignant cell inoculation. Mice treated with high doses of CP alone experienced an important weight decrease and died within the same time range as control mice (fig 6). In contrast, mice given high doses of CP + BLR lost very little weight and generally survived for > 3 mos. The same results were obtained with male BALB/c mice bearing lymphoma YC 8 ascitic cells. These data show that BLR can protect animals against the toxic effect of CP without impeding the anticancer activity of the drug.

DISCUSSION

Data described in this paper show that short-chain RNA molecules (termed BLR), obtained by mild degradation of purified *E. coli* r-RNA(s) with pancreatic RNase, promote leukocytogenesis in rabbits treated with CP. The particular base ratio of BLR (G + A/C + G = 2.35) is essential for their biologic activity on blood cells and for their priming effect in DNA synthesis. Intact r-RNA, synthetic polymers, BLR treated with alkali, or RNase A and T_1 are inactive or slightly active in bone marrow DNA synthesis.

It is well-known that this anticancer drug, one of the most widely used alkylating agents both in experimental and human therapy, damages rapidly dividing blood cells. BLR were expected to act on blood cell formation in living animals because in vitro, in the presence of DNA-dependent DNA polymerase I, they act as primers for the replication of DNA(s) isolated from rabbit bone marrow and spleen, and in live animals they concentrate to a large extent in the stem cell-containing tissues. When administered iv to CP-treated rabbits, BLR act by restoring normal levels of both granulocytes and lymphocytes in the circulating blood; they are eliminated a few days after injection. One could object that BLR probably act by liberating leukocytes stored in blood vessels and in some tissues. If this were so, one would expect that in rabbits undergoing prolonged treatment with very high CP doses, several consecutive administrations of BLR would exhaust the leukocyte stock, thus creating a situation in which the animals would die. This is not the case, and BLR injected iv result in an optimal increase in the

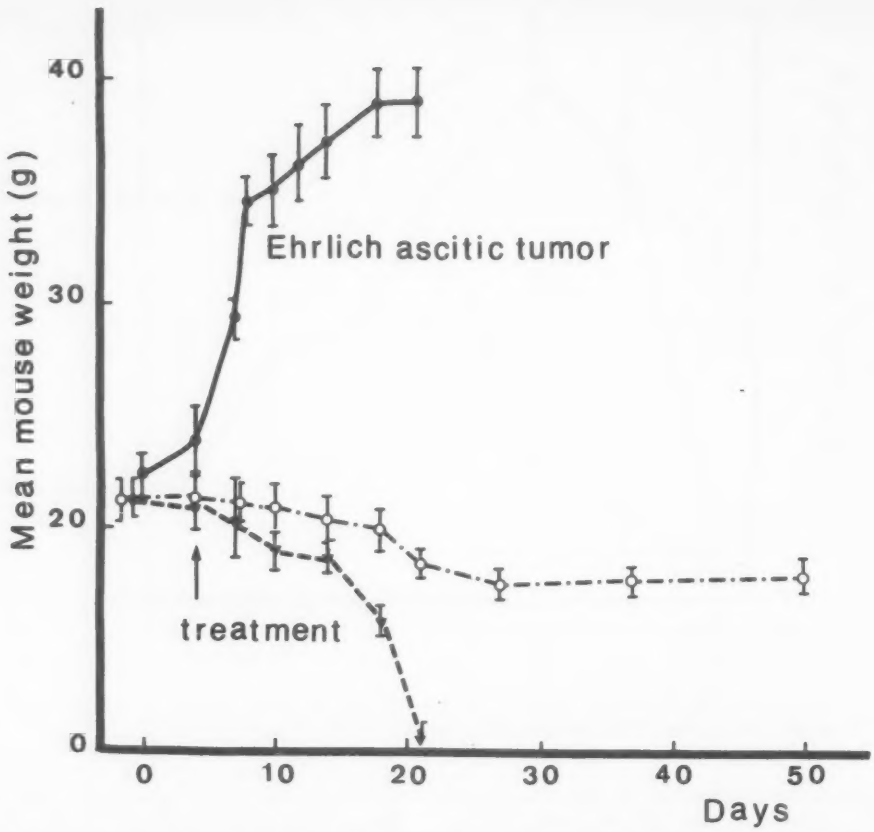

FIGURE 6.—Evolution of Ehrlich ascitic tumor cells in mice treated with CP or CP + BLR. Male Swiss mice (20-22 g) were inoculated ip with Ehrlich ascitic tumor cells (5×10^5) on Day 0, then divided into 3 groups of 10 animals each. The control group (solid circles) received physiologic saline solution. Another group (triangles) received CP for 15 consecutive days starting from Day 3: 2 mg iv twice daily (4-mg daily dosage). Another group (open circles) was given CP under identical conditions and received 1 mg of BLR ip every 4th day until CP was discontinued. Weight and survival duration of the animals were noted during and after treatment. In the control and the CP-treated groups no animal survived after Day 24. In the group treated with CP + BLR, 100% of the animals survived beyond Day 50.

leukocyte count which follows a weekly cycle and can be renewed, as demonstrated in rabbits continuously treated with large CP dosages (23-35 mg/kg/day for up to 3 mos). The CP dose level used in such experiments proves lethal within 13 days for many sensitive animals, but death is prevented by treatment with BLR. Thus, rabbits continuously treated with CP and receiving weekly BLR doses of 0.3-2 mg/kg (iv) have been kept alive and healthy for > 1-2 yrs. They have shown no ill effect after cessation of both antimitotic drug and BLR.

The physiologic effect of BLR in leukopoiesis is essentially based on three main observations. First, in both CP-treated and untreated rabbits, the BLR-increased leukocyte count was always found to be within physiologic limits. Second, BLR administered iv to CP-treated rabbits rapidly restored the CP-induced upset in the balance between granulocytes and lymphocytes. This balance-restoring effect and the following increase in both types of cells in circulating blood suggest that BLR not only influence the division process of stem cells but also act on stem cell derivatives which, in the multiplying compartment, differentiate into cells which give rise to granulocytes. Third, a large number of iv injections of BLR to normal or CP-treated rabbits do not lead to the appearance of a tolerance phenomenon such as is observed, for example, with bacterial endotoxin. Data presented elsewhere (20) show that in rabbits the physiologic activity of BLR on leukopoiesis can be easily distinguished from the effect of endotoxin which leads to temporary liberation of granulocytes and induces a tolerance phenomenon. In addition BLR are active on platelet formation (8), while endotoxin is not. When rabbits are treated with

high doses of CP over a long period, the animals can survive in good health only if they receive BLR at regular intervals. In view of the fact that BLR do not impede the anticancer effect of CP in cancer cell-bearing mice, BLR stand out as a choice blood-restoring compound for the treatment of tumors. Produced on a large scale, BLR may be of great help, not only in chemotherapy but also in correcting naturally occurring human leukocyte and platelet deficiencies.

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Use of Mathematical Models for the Evaluation of Two- and Three-Drug Combination Chemotherapy in Murine Tumor Models[1]

I. Fichtner* and G. Steinhoff[2,3]

In parallel with an ongoing clinical trial in patients with metastatic mammary carcinoma, we compared the three-drug combination of cyclophosphamide, tegafur, and methotrexate with the two-drug combination of cyclophosphamide and tegafur in the murine L1210 leukemia and B16 melanoma models. The obtained data based on a central composite design were calculated by mathematical models, which estimate optimum dose combinations for survival by restricting side effect constraints (loss of weight, leukopenia). The main results were as follows: (*a*) long-term survival can be reached only by accepting a relatively high toxicity in each case, and (*b*) in the L1210 model the three-drug combination was superior to the two-drug combination, while in the B16 melanoma cyclophosphamide as a single agent offered the best results. The experimental yields are in accordance with preliminary results of the clinical trial. The mathematical model used offers the possibility of comparing drug combinations in a relatively economic way. [Cancer Treat Rep 67:621–629, 1983]

During the past years the treatment of tumors in man with combinations of chemotherapeutic agents has gained growing importance. Most combinations used have been developed through the process of rational empiricism on the basis of drugs with different biochemical, cell cycle-specific, pharmacokinetic, and toxicologic profiles. However, only up to about 20% of all possible active two-drug combinations for a certain solid or hematologic malignancy have been tested clinically (1). Testing every combination would be impossible in clinical practice. Therefore, a lot of oncologists emphasize the necessity of better experimental models for predicting how to combine drugs more effectively (2,3). The advantages of such animal models are the relatively short time required to obtain results and the possibility of controlling all aspects of each trial whereas in humans an absolute control of variables is limited by ethical and moral constraints (3). Since our knowledge about the mechanism of drug interactions on the cellular level is limited even in animal models, an empiric approach is necessary (4). In general, a therapeutically synergistic combination can be defined according to Corbett et al (3) "as one that produces a therapeutic response which is superior to the maximum response obtainable by either agent alone at equitoxic doses." Therefore, not only therapeutic effectiveness but also toxicologic criteria must be registered during an experiment and must be included in the analysis. It is possible to reduce the number of test groups by using powerful statistical experimental designs and computer programs that locate the region of an optimum activity within certain response surfaces (5,6). Methods for determining optimum treatment levels of three-drug combinations including side effect constraints have been proposed and tested by Wampler et al (7) and Carter et al (8). It was our task to test the three-drug combination of cyclophosphamide (CPM), tegafur (Ftor), and methotrexate (MTX) in comparison with the two-drug combination of CPM and Ftor, in parallel with an ongoing controlled therapeutic trial in patients with metastatic mammary carcinoma. The three-drug combination was developed according to the CMF combination of Bonadonna et al (9) with Ftor substituted for 5-FU, which is possible without loss of any clinical efficacy (10). If it were possible to forgo MTX, the two-drug combination would be more appropriate for ambulatory adjuvant treatment.

We tested the combinations in two animal tumor models and included body weight reduction and leukocyte depression as side effect constraints. In each case, we used the so-called central composite design (7) and computer programs (11) to estimate the dose-response surfaces and to determine the optimum drug combinations.

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MATERIALS AND METHODS

Animal Models and Drugs

As experimental models we used the murine L1210 leukemia and B16 melanoma, both growing in female C57BL/6 × DBA/2 (BDF_1 hybrid) mice.

Leukemic cells (5×10^5), as a suspension in Hanks' balanced salt solution, were transplanted iv. The three- or two-drug combination was administered ip on Day 1. The percentages of mice living at least 12 or 15 days were determined. As toxic parameters the percentages of mice with a body weight loss $\geq 5\%$, $\geq 10\%$, and $\geq 15\%$ and a leukocyte count $\leq 5.0 \times 10^9$ and $\leq 2.0 \times 10^9$ cells /L were registered on Day 5. These values were used for mathematical modeling. Moreover, T/C values of the survival time for treated (T) mice compared with control (C) mice were also calculated.

The B16 melanoma was transplanted im as a diluted (1:5) tissue homogenate (0.1 ml/leg). Ip drug treatment followed on Days 1, 5, and 9. For mathematical modeling, the percentages of surviving mice on Days 30 and 35 and the percentages of body weight loss and leukocyte counts (corresponding to the L1210 model) on Day 12 were registered. Tumor volume inhibition and survival times (as T/C values) again served as additional therapeutic parameters. CPM (VEB Jenapharm), Ftor (V/O Medexport UdSSR), and MTX (Lederle) were dissolved or diluted immediately before use. The applications were carried out within 1 hour. Each group consisted of ten mice. Each experiment included one control group treated with physiologic saline. In confirmatory experiments the estimated optimum drug combinations were tested in the corresponding tumor model under the same conditions described above.

Mathematical Model

For the probability of surviving at least d days (P_d) after treatment with our three-drug combination, we used an adequate mathematical model that was described by Wampler et al (7). This model is given by

$$P_d = (1-\exp(-\underline{x}'\underline{\beta}))^{-1},$$

where

$$\underline{x}'\underline{\beta} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1^2 + \beta_5 x_2^2 + \beta_6 x_3^2 + \beta_7 x_1 x_2 + \beta_8 x_1 x_3 + \beta_9 x_2 x_3$$

and

$$x_1 = \frac{\ln(\text{dose of MTX in mg/kg}) - \ln 10}{10}$$

$$x_2 = \frac{\ln(\text{dose of Ftor in mg/kg}) - \ln 80}{10}$$

$$x_3 = \frac{\ln(\text{dose of CPM in mg/kg}) - \ln 40}{10}.$$

According to this scaling the experiment is centered in the logarithmic scale at 10 mg/kg of MTX, 80 mg/kg of Ftor, and 40 mg/kg of CPM and all x values lie in the interval (-1, 1). Our experimental data are limited as follows:

$$\begin{aligned} 0 < \text{MTX} &\leq 45 \text{ mg/kg} \\ 0 < \text{Ftor} &\leq 360 \text{ mg/kg} \\ 0 < \text{CPM} &\leq 180 \text{ mg/kg}. \end{aligned}$$

Furthermore, we used the central composite design that was also described by Wampler et al (7). We could slightly improve the goodness of fit of the estimated dose-response surfaces by using nine additional points of design. Our complete design can be seen in table 1: the observed and the predicted outcomes of treatment for each of the treatment groups of the three-drug combination in the L1210 model and the corresponding sums of square deviations (SSD). We used the very same design for the estimation of the dose-response surfaces of the B16 melanoma and got a similar good adequacy (table 2).

The mathematical model for probability of realizing the restriction that weight loss must not be more than w (P_w) is analogous to P_d:

$$P_w = (1-\exp(-\underline{x}'\underline{\gamma}))^{-1}.$$

The model for the probability of realizing the restriction that the leukocyte count has to be greater than L (P_L) is similarly given by

$$P_L = (1-\exp(-\underline{x}'\underline{\vartheta}))^{-1}.$$

Since a transformation of the above models into quasi linear regression models would effect a great bias of our experimental data, the β_i, γ_i, and ϑ_i ($i = 1, 2, \ldots, 9$) in the above nontransformed models were estimated by the computer program RESPI (11) for nonlinear regression based on the spiral algorithm (12). Under the restriction that the probability (P_w, P_L) of realizing the side effect constraint has to be at least a given level (table 3), the maximum of the estimated function P_d (table 3) was determined by the computer program CREEP (11) based on a random-search algorithm. This means that during the whole procedure the dependence between the weight loss or leukocyte counts on the one hand and the survival time on the other hand was always simultaneously considered. The mathematical models for P_d, P_w, and P_L for the two-drug combination follow from the above models by deleting the variable x_1. Our two-drug design (without the single agents) corresponds to the above-mentioned experimental design for the three-drug combination. Here we also got a sufficient goodness of fit.

As far as we know, in our case there are no suitable statistical tests of the goodness of fit, because for the number N of animals in the respective treatment groups all magnitudes $N\hat{P}_\alpha(i)\,(1-\hat{P}_\alpha(i))$ are too small, where $\hat{P}_\alpha(i)$ is the estimated probability of favorable outcome in the i^{th}

TABLE 1.—Central composite design: observed and predicted treatment results for the 3-drug combination in the L1210 model

Group No.	Dose (mg/kg)			No. of animals*													
				Surviving at least—				With weight loss—						With leukocyte counts (× 10^9/L)—			
				12 days		15 days		⩽5%		⩽ 10%		⩽ 15%		⩾ 5.0		⩾ 2.0	
	CPM	Ftor	MTX	o	p	o	p	o	p	o	p	o	p	o	p	o	p
1	0	0	0	0	0.0	0	0.0	10	10.0	10	10.0	10	10.0	10	10.0	10	10.0
2	16	32	4	2	0.0	0	0.0	10	10.0	10	10.0	10	10.0	10	10.0	10	10.0
3	100	32	4	1	1.0	1	0.1	1	2.7	8	9.0	9	10.0	2	2.0	9	9.4
4	16	32	25	0	0.0	0	0.0	10	9.9	10	10.0	10	10.0	10	10.0	10	10.0
5	16	200	4	0	0.1	0	0.0	10	9.8	10	9.7	10	9.9	10	10.0	10	9.9
6	100	32	25	0	0.3	0	0.1	2	1.0	8	8.2	9	9.3	1	1.0	7	7.0
7	100	200	4	10	9.8	1	1.0	0	0.5	7	6.9	10	9.7	1	0.0	6	6.0
8	16	200	25	0	0.3	0	0.0	10	9.9	10	10.0	10	9.8	10	10.0	10	9.8
9	100	200	25	9	9.3	6	6.0	2	1.0	9	9.3	10	9.6	0	0.0	2	2.0
10	180	80	10	10	9.7	6	6.0	4	0.2	8	7.6	10	9.8	0	0.0	4	4.0
11	8	80	10	0	0.0	0	0.0	10	10.0	10	10.0	10	10.0	6	10.0	10	10.0
12	40	80	45	1	0.4	0	0.0	7	7.9	10	10.0	10	9.9	7	7.1	9	9.1
13	40	80	2	0	0.3	0	0.0	9	8.0	10	9.9	10	10.0	9	9.5	10	9.9
14	40	80	10	0	0.1	0	0.0	10	8.7	10	9.9	10	9.9	10	9.4	10	9.6
15	40	360	10	6	6.0	0	0.2	5	5.5	6	6.1	7	7.1	0	0.1	5	5.0
16	40	16	10	0	0.0	0	0.0	9	8.5	10	9.3	10	9.6	10	10.0	10	9.9
17	40	0	0	0	0.0	0	0.0	10	9.6	10	10.0	10	10.0	7	8.2	10	10.0
18	100	0	0	10	10.0	0	0.1	6	6.4	9	10.0	10	10.0	8	6.2	10	9.9
19	180	0	0	10	10.0	6	6.0	3	2.6	9	9.9	9	9.0	2	3.2	9	9.0
20	0	80	0	0	0.0	0	0.0	9	10.0	10	10.0	10	10.0	10	9.9	10	10.0
21	0	200	0	0	0.0	0	0.0	10	10.0	10	10.0	10	10.0	10	10.0	10	10.0
22	0	360	0	1	1.0	0	0.1	9	9.0	10	10.0	10	10.0	10	10.0	10	10.0
23	0	0	10	0	0.0	0	0.0	10	10.0	10	10.0	10	10.0	10	9.9	10	10.0
24	0	0	25	0	0.0	0	0.0	10	10.0	10	9.9	10	10.0	10	10.0	10	10.0
25	0	0	45	0	0.0	0	0.0	8	8.0	9	9.0	9	9.0	10	10.0	10	10.0
SSD					4.9		2.0		24.9		2.9		1.5		23.7		0.4

* o = observed; p = predicted.

TABLE 2.—Central composite design: observed and predicted treatment results for the 3-drug combination in the B16 model

Group No.	Dose (mg/kg)			No. of animals*													
				Surviving at least—				With weight loss—						With leukocyte counts (× 10^9/L)—			
				30 days		35 days		⩽ 5%		⩽ 10%		⩽ 15%		⩾ 5.0		⩾ 2.0	
	CPM	Ftor	MTX	o	p	o	p	o	p	o	p	o	p	o	p	o	p
1	0	0	0	0	0.0	0	0.0	10	10.0	10	10.0	10	10.0	10	10.0	10	10.0
2	16	32	4	0	0.0	0	0.0	9	9.6	10	10.0	10	10.0	9	9.4	10	10.0
3	100	32	4	10	9.5	4	4.0	2	2.7	5	6.9	7	7.9	0	0.4	0	0.2
4	16	32	25	0	0.0	0	0.0	6	6.6	8	7.8	9	8.1	6	6.2	9	8.7
5	16	200	4	0	0.0	0	0.0	8	8.5	9	9.9	10	10.0	1	1.2	9	9.9
6	100	32	25	6	5.9	5	5.0	0	0.8	2	0.2	2	2.1	0	0.1	0	0.1
7	100	200	4	7	6.9	4	4.0	4	0.6	7	5.0	8	7.6	0	0.3	0	0.0
8	16	200	25	0	0.0	0	0.0	7	5.8	9	7.9	9	9.0	3	2.9	10	9.9
9	100	200	25	0	0.0	0	0.1	0	0.4	0	0.3	0	0.4	0	0.2	0	0.0
10	180	80	10	2	2.2	2	2.0	0	0.3	0	0.6	2	1.0	0	0.1	0	0.0
11	8	80	10	0	0.0	0	0.0	7	9.6	10	10.0	10	10.0	10	9.5	10	10.0
12	40	80	45	1	0.0	0	0.0	0	1.0	0	0.7	0	0.9	1	1.3	6	6.3
13	40	80	2	0	0.4	0	0.0	5	5.0	9	9.8	9	10.0	2	1.8	9	9.0
14	40	80	10	1	0.0	1	0.0	5	5.0	7	7.7	8	8.4	2	2.0	9	9.0
15	40	360	10	0	0.0	0	0.0	2	2.9	4	5.8	9	9.4	0	0.1	1	1.1
16	40	16	10	0	0.4	0	0.0	8	7.3	9	7.0	10	9.2	2	1.4	10	9.8
17	40	0	0	0	0.6	0	0.5	8	8.0	10	8.8	10	9.3	10	9.9	10	10.0
18	100	0	0	8	7.6	6	5.7	1	1.1	3	4.9	7	7.5	0	0.1	1	1.1
19	180	0	0	5	5.2	3	2.7	1	0.1	4	2.2	6	5.8	0	0.0	0	0.0
20	0	80	0	0	0.0	0	0.0	10	9.9	10	10.0	10	10.0	10	10.0	10	10.0
21	0	200	0	0	0.0	0	0.0	10	9.5	10	9.8	10	10.0	10	10.0	10	10.0
22	0	360	0	0	0.0	0	0.0	8	8.2	8	8.0	10	10.0	10	10.0	10	10.0
23	0	0	10	0	0.0	0	0.0	10	10.0	10	9.9	10	10.0	10	10.0	10	10.0
24	0	0	25	0	0.0	0	0.0	7	7.0	8	7.6	10	9.8	10	10.0	10	10.0
25	0	0	45	0	0.0	0	0.0	0	0.2	1	1.6	8	8.0	3	2.5	10	10.0
SSD				3.2		1.4		27.8		56.1		6.5		0.8		2.4	

*o = observed; p = predicted.

TABLE 3.—Estimated optimum doses of the 3-drug combination in the L1210 model for at least a 15-day survival time, under the declared restrictions*

Side effect constraint		Probability of realizing the constraint		Estimated optimal doses (mg/kg)			Estimated probability of mice living ⩾ 15 days
Weight loss (%) no more than	Leukocyte count (10^9/L) less than	Required at least	Estimated	CPM	Ftor	MTX	
5		0.85	0.85	33	360	45	0.20
		0.90	0.90	29	360	45	0.15
		0.95	0.95	24	360	45	0.10
		0.85	0.91	180	360	45	1.0
10		0.90	0.91	180	360	45	1.0
		0.95	0.95	180	246	45	0.99
		0.85	0.95	180	360	45	1.0
15		0.90	0.95	180	360	45	1.0
		0.95	0.95	180	356	45	1.0
		0.85	0.85	17	360	45	0.05
	5	0.90	0.90	16	360	45	0.04
		0.95	0.95	15	360	45	0.04
		0.85	0.85	180	82	2	0.30
	2	0.90	0.90	180	50	2	0.18
		0.95	0.95	12	360	45	0.03

* Mean survival time of the control mice was 6.5 days after iv inoculation of 5×10^5 tumor cells.

group [for example, *see also* page 40 of (13); or (14)]. However, by our calculated sums of SSD the goodness of fit can be compared well between the treatment groups and also between different experiments.

RESULTS

An example for estimated optimum doses of the three cytostatics used in combination, depending on different required probabilities for achieving side effect constraints, is given in table 3. First, it can be seen that the mathematical model considers the required side effect constraints. The higher the demand (corresponding to lower toxicity), the lower the doses of the effective drugs. This must be "paid for" by lower probabilities for surviving a certain day. Thus, the demand of 15-day lifespan in the L1210 model can be realized only if a relatively high toxicity (weight loss $\leqslant$ 10% and $\leqslant$ 15%) is accepted. If only one optimum for the realization of a certain constraint could be found, then the higher the required probability for realizing side effect constraints, the lower the doses for the most critical drugs (eg, Ftor for weight loss and CPM for leukopenia). In the case of the constraint that the leukocyte count not be $< 2 \times 10^9$ cells/L, two optimum regions were found, which realized the constraint with different probabilities.

Table 4 shows the estimated optimum doses of the three-drug combination directly compared with the two-drug combination for the certain required side effect constraints in the L1210 model. The required survival time should be 15 or 12 days. The control mice died between Day 6 and Day 8 after tumor inoculation. It can be seen that the demand of at least a 15-day survival time cannot be achieved with low toxic effects (weight loss $\leqslant$ 5%, leukocyte count $\geqslant 5.0 \times 10^9$/L). In most cases, higher estimated probabilities for obtaining the demanded survival time within one constraint could be reached for the three-drug combination. The same tendencies can be seen for the demand of at least a 12-day survival time, though in general higher probabilities could be noted. Groups 4, 6, and 14, with the maximum doses of CPM and extremely low doses of Ftor, also estimate a relatively good chance of survival, while groups with high doses of Ftor and low doses of CPM (Nos. 8 and 18) realize very low toxic effects but with minimum chances of survival. In most cases the mathematical model gave correct predictions, with differences of ± 10% (one animal per group) being considered as accurate. Also, some larger differences could be explained in each case by biologic host variabilities; for instance, the leukocyte counts of Groups 7 and 17 (table 4) were narrowly < 5.0 but not, as estimated in 95% of cases, 5.0×10^9/L.

In the B16 melanoma model, the control mice died between Day 18 and Day 22 after tumor inoculation. As shown in table 5, a required survival time of 35 days can be obtained only with the three-drug combination by accepting a high toxicity ($\leqslant$ 15% weight loss). The chance of a 30-day survival period, too, is in most cases higher with the three-drug combination compared with the two-drug combination. However, the dose of MTX in the three-drug combination is always 2 mg/kg, the lowest dose that was allowed to be estimated by the mathematical model. As confirmatory experiments showed, this small dose plays no role in gaining any therapeutic or toxicologic effect.

The optimum doses of the two-drug combinations, with

TABLE 4.—Estimated optimum doses of the 3- and 2-drug combination in the L1210 model for at least 95% probability for achieving the required side effect constraint, and results of the corresponding confirmatory tests *

Group No.	Survival time at least (days)—	P_d	Required weight loss (%) no more than—	P_w	Required leukocyte count (10^9/L) no less than—	P_L	Estimated optimum doses (mg/kg) CPM	Ftor	MTX	Mean survival time (days)	T/C (%)	15-day survivors	Mean Leukocyte count (10^9/L)	Mean body weight difference (%)
1	15	0.10	5	0.95			24	360	45					
2	15	0.0 †	5	0.95†			53	46		10.8	171	0	3.7	0
3	15	0.99†	10	0.95†			180	246	45	29.4	414	3	0.6	-5
4	15	0.89†	10	0.99†			180	16		16.7	265	0	0.79	-6
5	15	0.99†	15	0.95†			180	356	45	24.1	354	3	0.22	-10
6	15	0.89†	15	0.99†			180	16		16.7	256	0	0.79	-6
7	15	0.04†			5	0.95‡	15	360	45	10.1	142	0	3.9	-5
8	15	0.0 †			5	1.0 †	8	360		9.8	138	0	5.7	-2
9	15	0.03			2	0.95	12	360	45					
10	15	0.0			2	0.95	91	56						
11	12	0.61	5	0.95			24	360	45					
12	12	0.87†	5	0.95†			43	360		12.4	197	0	2.9	-3
13	12	1.0 †	10	0.95†			180	246	45	29.4	414	3	0.6	-5
14	12	1.0 †	10	0.95†			180	29		13.7	204	0	0.5	-4
15	12	1.0 †	15	0.95†			180	320	2	24.6	346	2	0.3	-6
16	12	1.0 †	15	0.95†			80	360		14.6	206	0	1.4	-6
17	12	0.44†			5	0.95‡	15	360	45	10.1	142	0	3.9	-5
18	12	0.02†			5	0.95†	14	360		9.8	138	0	5.7	-2
19	12	0.88			2	0.95	98	80	2					
20	12	0.88			2	0.95	83	104						

* P_d, P_w, P_L = estimated probability of achieving the side effect constraints lifespan, weight loss, and leukocyte count.
†,‡Correct (†) and incorrect (‡) prediction by the mathematical model.

TABLE 5.—Estimated optimum doses of the 3- and 2-drug combination in the B16 melanoma for at least 95% probability for achieving the required side effect constraint, and results of the corresponding confirmatory tests*

Group No.	Survival time at least (days)—	P_d	Required weight loss (%) no more than—	P_w	Required leukocyte count (10^9/L) no less than—	P_L	Estimated optimum doses (mg/kg/day) CPM	Ftor	MTX	Mean survival time (days)	T/C (%)	Tumor volume inhibition (%)	Mean leukocyte count (10^9/L)	Mean body weight difference (%)
1	35	0.0	5	0.95			24	16	2					
2	35		5				N	N						
3	35	0.25	10	0.95			68	46	2					
4	35	0.0	10	0.95			28	16						
5	35	0.99‡	15	0.95			110	360	2	26.3	126	97	0.3	-16
6	35	0.0 †	15	0.95			32	16		26.8	133	80	4.0	-5
7	35	0.0 †			5	0.95	21	16	2	22.1	106	7	9.4	3
8	35	0.0 †			5	0.95†	22	16		24.4	117	5	8.1	2
9	35	0.08†			2	0.95†	55	16	2	27.5	132	82	3.1	-2
10	35	0.03			2	0.95	40	26						
11	30	0.0 †	5	0.95†			24	16	2	20.3	97	7	9.4	3
12	30		5				N	N						
13	30	0.99‡	10	0.95†			72	16	2	29.4	141	91	1.8	-3
14	30	0.29†	10	0.96†			8	360		22.9	113	42	4.8	-1
15	30	0.99‡	15	0.95‡			110	360	2	26.3	126	97	0.3	-16
16	30	0.29†	15	0.98†			8	360		22.9	113	42	4.8	-1
17	30	0.0 †			5	0.95†	21	16	2	22.1	106	7	9.4	3
18	30	0.29†			5	0.99‡	8	360		22.9	113	42	4.8	-1
19	30	0.91‡			2	0.95†	55	16	2	27.5	132	82	3.1	-2
20	30	0.29†			2	0.99†	8	360		22.9	113	42	4.8	-1

*P_d, P_w, P_L = estimated probability of achieving the side effect constraints lifespan, weight loss, and leukocyte count. N = no point could realize the required side effect constraint.
†,‡Correct (†) and incorrect (‡) prediction by the mathematical model.

a mean chance of surviving 30 days, was always 8 mg/kg/day of CPM plus 360 mg/kg/day of Ftor, showing that Ftor is well-tolerated (low toxicity) in very high repeated doses. However, the therapeutic efficacy in this model is not very convincing. The realization of the required side effect constraints and chances of survival were tested in confirmatory experiments and in most cases the demands required were met (table 5). If a 90% probability of a 30- or 35-day survival time was predicted (table 5, Groups 5, 13, and 15), this could not be proved by the confirmatory experiments because of the high trial-to-trial variability of the B16 tumor model. But these groups really showed the highest mean survival times and therefore confirmed the correct predictions in principle.

DISCUSSION

It is somewhat difficult for an experimentalist to meet the growing demands of clinical oncologists for animal models that are highly predictive of optimum drug combinations for human tumors. An optimal drug dosage in single therapy is not the same in a combination regimen because many drug-drug interactions on different fields of pharmacologic activity are possible (15). To test all of these possibilities in a simple numerical way would be too time- and material-consuming. On the other hand, a direct comparison of two combinations or of one combination with the single-drug therapy is possible only on the basis of nearly equitoxic levels. We think that these difficulties can be overcome by the help of a mathematical model, as was proposed by Wampler et al (7). In a relatively economic way, the model offers the possibility not only of optimizing a given drug combination but also of comparing combinations on the basis of certain required side effect constraints. The estimated optimum doses of the combinations were tested in confirmatory experiments, and in the L1210 model, we obtained T/C values that could never be reached with the conventional method of calculating experimental dosages from clinical data on a milligram per meter squared basis (16). With that method, we achieved the best results (high T/C values with acceptable toxicity) with CPM alone (185 mg/kg on Day 1 only): 272% T/C, 2.3 × 10^9 wbcs/L, 3% body weight loss, and one of ten animals cured. As shown in the confirmatory tests in the murine leukemia, still higher T/C values with more cures could be obtained by optimizing the three-drug combination. However, here, too, one disadvantage of the mathematical model becomes obvious. Though the required constraints are realized with a high probability in almost each case, not always exactly equitoxic dosages result, because the realization occurs with each combination at a different degree. The somewhat contrary results we got in the B16 melanoma are based on the relative resistance of this tumor to MTX and its only slight sensitivity to Ftor. With CPM alone (100 mg/kg/day) we obtained the best therapeutic results: 158% T/C, 1.0 × 10^9 wbcs/L, and 12% body weight loss. Therefore, this model does not correspond to the clinical mammary carcinomas which are generally sensitive to all three drugs, and the obtained experimental results cannot be transferred to clinical practice. For further testing of a combination chemotherapy, only such animal models should be selected which have similar drug sensitivities as the corresponding human tumors. It is only rarely possible to use animal experiments to propose the exact dose combination for clinical use, because such factors as recovery time of certain toxicologic effects, different multiple drug schedules, the appearance of resistant cells, and the difference in cellular and growth kinetics between experimental and human tumors cannot be considered simultaneously within one certain group of experiments. However, we are of the same opinion as van Putten (4) that it is possible to draw some conclusions about tendencies which can be extrapolated to clinical practice.

In view of our experiments with the more relevant model L1210, we would propose the three-drug combination in nearly maximally tolerated doses for clinical use. For repeated use, as is common in clinical practice, the doses of CPM and MTX should be reduced, while Ftor can be given repeatedly in large amounts. Instead of the two-drug combination, we think that CPM alone has comparable therapeutic effects with lower toxicity.

Preliminary results of the controlled therapeutic trial on patients with metastatic mammary carcinoma in our clinic are not inconsistent with our results. While the 15 patients treated with the three-drug combination (15 mg/kg of CPM + 25 mg/kg of Ftor + 0.5 mg/kg of MTX on Days 1, 8, and 15) showed a 30% remission rate with a median duration of 8.5 months, the 11 patients treated with the two-drug combination (10 mg/kg of CPM + 25 mg/kg of Ftor once/week, for 10 weeks) showed only an 18% remission rate with a median duration of 4 months (M. L. Matthes, personal communication).

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Inhibition of Methionine Transport by Methotrexate in Mitogen-Stimulated Human Lymphocytes[1,2]

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Mitogen stimulation of human lymphocytes was used to study the expression of several amino acid transport systems in response to cell growth. Resting lymphocytes possess detectable neutral amino acid transport systems which can be activated by the mitogen, phytohemagglutinin (PHA). Sodium-independent transport of 2-aminobicyclo (2,2,1) heptane 2-carboxylic acid was similar in resting and stimulated lymphocytes. However, sodium-dependent uptake of methyl aminoisobutyric acid and α-aminoisobutyric acid increased threefold to eightfold with stimulation by PHA. Methionine uptake was primarily sodium-independent in resting lymphocytes, whereas methionine uptake increased fourfold to eightfold in stimulated cells; most of this increase was attributable to sodium-dependent transport. Uptake of methotrexate (MTX) was minimal in resting lymphocytes but was maximal 72 hrs after stimulation with PHA; this increase in MTX uptake was accompanied by inhibition of sodium-dependent methionine transport. These results suggest that PHA activates sodium-dependent transport of methionine and that this activation is blocked by the folate antagonist, MTX. [Cancer Treat Rep 67:631–639, 1983]

Mitogen stimulation of normal human lymphocytes is a common method for studying the activation of resting cells (1–5) and is accompanied by increased nutrient uptake (2). However, the mechanism(s) by which the transport systems are activated is poorly understood.

Most mammalian cells have at least three neutral amino acid transport systems: the "A," "L," and "ASC" systems. These systems are responsible for the cellular transport of amino acids. The alanine or "A" system, which is dependent on extracellular sodium, is specific for the transport of the nonmetabolizable substrate, methyl aminoisobutyric acid (MeAIB) (6,7). The leucine or "L" system is characterized by its lack of dependence on extracellular sodium and its specificity for the nonmetabolizable substrate, 2-aminobicyclo (2,2,1) heptane 2-carboxylic acid (BCH) (6,8). The "ASC" system is less well known and may have different properties between cells (9,10). This system has been shown to make a major contribution to concentrating essential amino acids for mammalian cells in culture (11). Recently, surprising differences in the substrate specificity have also been demonstrated for the ASC system between normal rat hepatocytes and the methionine auxotrophic hepatoma cells, HTC (12). Mitogen-stimulated human lymphocytes are also methionine auxotrophic cells (13).

Amino acid transport has been studied in several species of activated lymphocytes (1–5,14–17), but a complete study of the changes in transport in response to initiation of cell growth has not been carried out. Methionine is an essential amino acid and an absolute growth requirement for activated human lymphocytes and some tumor cells (13,18–20); it is known to be both regulated by and dependent upon folate biosynthesis (21). The present study was initiated to determine whether methotrexate (MTX), a 2,4-diamino folate antagonist and an inhibitor of dihydrofolate reductase (22–24), has any effect on the cellular transport of methionine. In addition, we hoped to discern any possible relationship between folate (one-carbon) metabolism and methionine transport at the membrane level. This work has previously been reported in abstract form (15).

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MATERIALS AND METHODS

Reagents

3′,5′,7-[^{3}H]MTX (205 mCi/mmol) and [G-^{3}H] threonine (309 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). BCH (4.95 mCi/mmol), α-aminoisobutyric acid (AIB) (53.2 mCi/mmol), and MeAIB (51.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA). MTX was obtained from Lederle Laboratories (Pearl River, NY).

Lymphocytes

Venous blood was obtained from normal volunteers, in heparinized containers (14 units/ml). The peripheral blood was diluted 1:1 (vol/vol) with sterile isotonic saline, and the mononucleated cells were isolated by Ficoll-Hypaque gradient centrifugation at room temperature for 40 mins at 400 × g (25). Mononuclear cells were harvested, washed three times with sterile saline, and resuspended in RPMI-1640 medium. The wbc count was determined by counting on a hemocytometer. Cell viability (98%) was measured by trypan blue exclusion. Morphologic characterization of the blast cells was determined by Jenner-Giemsa staining throughout these studies (1,25).

Lymphocytes were cultured at a density of 2 × 10^6 cells/ml in RPMI-1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin (Gibco, Grand Island, NY) in 12 × 75-mm culture tubes. The tubes were incubated at 37°C in 5% CO_2 for up to 96 hrs. The "T" lymphocyte-specific mitogen, phytohemagglutinin (PHA; 0.01 ml/ml; Gibco) was added to the culture tubes in duplicate and the cells were studied daily, for 4 days, for lectin responses. Cellular response to PHA was assessed by [^{3}H]thymidine incorporation into acid-precipitable material, by visual morphologic changes (26), and by cell volume changes as measured by a Coulter Counter Channelyzer.

DNA Synthesis

At each time point, 10^6 human lymphocytes were harvested and incubated in Earle's balanced salt solution (BSS) (Gibco) with 0.5 μCi/ml of [^{3}H]thymidine (5 Ci/mmol; New England Nuclear) at 37°C for 0, 15, 30, 45, and 60 mins. After incubation the cells were precipitated at 0°C with 5% perchloric acid (PCA) (vol/vol) and 1 mg of carrier bovine serum albumin. The precipitate was washed three times and the pellet was solubilized overnight in 1 ml of NCS (Nuclear of Chicago Solubilizer, Amersham) and 0.1 ml of water at 37°C. The solubilized precipitate was counted in 10 ml of scintillation fluid in a Beckman LS-250 liquid scintillation counter.

Amino Acid Transport Studies

Incubation medium-1 (IM-1) was Earle's BSS containing 116 mM NaCl. The monovalent cation concentration was concomitantly reduced for all incubations when any added substrate contributed > 10 mosm in the medium. Incubation medium-2 (IM-2) was a sodium-free Earle's BSS containing 116 mM choline chloride. This buffer was used to measure sodium-independent transport. The difference between the uptake in IM-2 and incubation medium was considered the net sodium-dependent transport.

Transport studies were done according to the following protocol: human lymphocytes were harvested by centrifugation, washed three times with the respective incubation medium, and resuspended in it at a final density of 5 × 10^6 cells/ml. The viable cell count was determined by trypan blue exclusion, using a hemocytometer. For each transport determination, cells were preincubated for 25 mins in a 37°C water bath. At zero time, the labeled amino acid and, if necessary, the competing substrate were added to the incubation medium at 37°C. At timed intervals, 200-μl samples were withdrawn and the cells were separated from the incubation medium by centrifugation through a discontinuous gradient of silicone-mineral oil into PCA. This technique is similar to the method of Wohlhueter et al (27).

In the present experiments, centrifugation was carried out using 400-μl conical polyethylene tubes (Sarstedt), which were prepared by layering 100 μl of silicone-mineral oil on top of 50 μl of 5% (vol/vol) PCA. Each 200-μl aliquot of cells withdrawn from the incubation medium was rapidly layered on top of the oil, and the tube was immediately centrifuged at 11,600 × g for 5 secs in a Beckman Microfuge B.

At the completion of centrifugation, aliquots of the PCA fraction were placed in 6 ml of Aquasol II and counted for 10 mins in a Beckman LS-250 liquid scintillation counter. The data presented in these figures are the mean of at least three experiments which included all drug treatments.

MTX Transport

Transport of [^{3}H]MTX (25 μM) was determined by incubating the cells at 1-5 × 10^7 cells/ml (IM-1). The reaction was stopped by pipetting a small aliquot (0.2 ml) of cell suspension into 20 volumes of cold normal saline (0.9% NaCl solution) and centrifuging for 5 secs in a Beckman Microfuge B. The cell pellet was washed twice with 100 volumes of normal saline and was dissolved in 0.5 ml of NCS and in 0.1 ml of H_2O overnight at 37°C. The samples were counted in scintillation fluid with a Beckman liquid scintillation counter.

To estimate the role of diffusion in the transport studies, each experiment with lymphocytes was duplicated at 0°C. The diffusion component was subtracted from the total transport to obtain net uptake.

Metabolism

Acid-Soluble Labeled Methionine.—[^{14}C]-1-methionine (60 mCi/mmol) was added to the cells as described above. The PCA-soluble fraction of the gradient was analyzed for metabolites of methionine. Aliquots from the soluble fraction of the transport reaction (10^6 cells) were diluted with *N*-butanol-acetic acid-water (2:1:4) and were spotted on Whatman 1-mM paper (46 × 75 cm) sheets. Descending paper chromatography was performed at room temperature for 14 hrs using *N*-butanol-acetic acid-water (2:1:1) as buffer. The chromatogram was dried and cut into 2-cm sections. Each sample was counted in 10 ml of scintillation fluid. Labeled (*a*) *S*-adenosylmethionine (SAM); (*b*) serine; (*c*) *S*-adenosyl homocysteine (SAH); (*d*) methionine sulfoxide; and (*e*) methionine were routinely employed as standards for chromatography with R_f values of 0.08, 0.12, 0.25, 0.45, and 0.68, respectively. Methionine sulfoxide was identified by ninhydrin (R_f = 0.45). For comparison, samples were also tested using high-performance liquid chromatography. Similar results were obtained.

Acid-Insoluble Material.—The acid-insoluble material (10^6 cells) containing labeled methionine was washed three times with 5% PCA, dried in 90% ethanol, and solubilized overnight in 0.5 ml of NCS and 0.1 ml of H_2O at 37°C. The solubilized pellet was counted in scintillation fluid (10 ml) with a Beckman LS-250 liquid scintillation counter.

RESULTS

Increase in Cell Volume Caused by Lymphocyte Stimulation With PHA

The cell volume of the stimulated lymphocytes increased to a maximum at 72 hrs after exposure (fig 1). Most of the cells had reduced their volume by more than one-half at 96 hrs after stimulation (fig 1). The cell volume of unstimulated lymphocytes remained unchanged through 96 hrs. Labeled thymidine incorporation into acid-precipitable material was also maximal at 72 hrs (fig 1).

Amino Acid Transport by Resting Lymphocytes

The uptake of MeAIB, a nonmetabolizable amino acid analog, by resting lymphocytes was detectable only in IM-1 and therefore was considered sodium-dependent (fig 2A; 0 time). The uptake of AIB and BCH, two other amino acid analogs, was also measured in resting lymphocytes and was found to be primarily sodium-independent (fig 2, B and C; 0 time). The uptake of methionine into these cells was primarily via sodium-independent transport (figs 2D and 3A).

In resting cells (10^6), most of the labeled methionine (95%) was found in the acid-soluble fraction during the first 5 mins. About 50% of the labeled material co-migrated with methionine sulfoxide on descending paper chromatography; the remainder was apparently methionine. In mitogen-stimulated (72 hrs) lymphocytes (10^6), > 90% of the labeled methionine co-migrated with unlabeled methionine. The amount of labeled material found

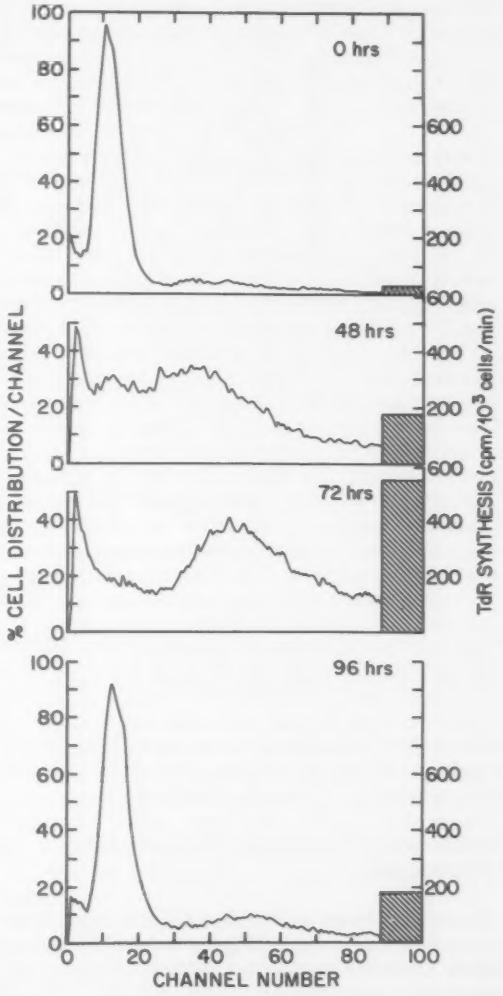

FIGURE 1.—Cell volume analysis and thymidine incorporation into acid-precipitable material in human lymphocytes. Normal resting (0 hr) and PHA-stimulated (49, 72, and 96 hrs) lymphocytes were analyzed on a Coulter Counter Channelyzer. Cell volume ranged from 2 to 4 cm³ (stimulated). At the same time points the cells were assayed for [^{3}H]thymidine incorporation into acid-precipitable material. *See* Materials and Methods section for details.

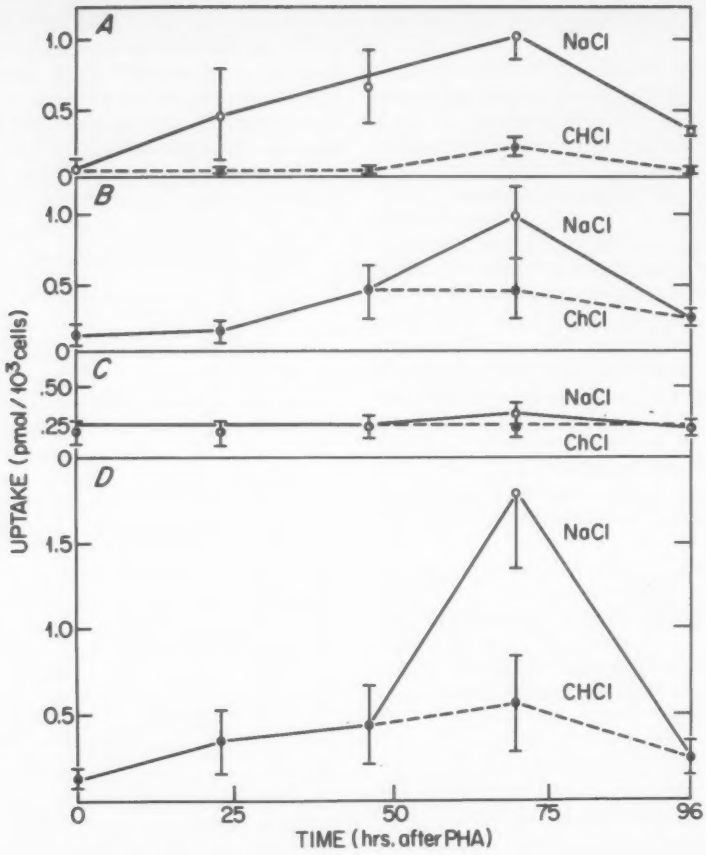

FIGURE 2.—Amino acid transport in lymphocytes. Cells were incubated in the presence of sodium chloride (○) or choline chloride (●). Uptake studies were measured with the labeled substrates: MeAIB (panel A), AIB (panel B), BCH (panel C), and methionine (panel D). Uptake was measured as pmol/10^3 cells/min and has been described in the Materials and Methods section.

in the acid-insoluble fraction from resting cells (10^6) or mitogen-stimulated cells (10^6) was < 5% of the total label taken up into the cell during the first 5 mins.

Amino Acid Transport by PHA-Stimulated Lymphocytes

The cellular uptake of MeAIB and AIB increased in response to stimulation with PHA (fig 2, A and B). This increase, primarily in sodium-dependent transport, occurred 48–72 hrs after stimulation when the cells had completed mitosis (96 hrs) and corresponded with maximum cell growth and DNA synthesis. There was no significant change in the initial sodium-independent uptake of BCH when the cells were stimulated by PHA (fig 2C).

Methionine uptake increased fourfold to eightfold 72 hrs after stimulation with PHA (fig 2D). Methionine uptake continued for 25–30 mins; however, a component of this is due to metabolism. This increase was primarily sodium-dependent and also coincided with maximum cellular growth and DNA synthesis. There was a corresponding decrease in methionine uptake upon the completion of mitosis (96 hrs). The metabolism of labeled methionine was minimal at 72 hrs (fig 4, B–E).

Reduction in Amino Acid Uptake by MTX in Stimulated Lymphocytes

MTX (10 μM) had no effect on methionine (500 μM) uptake in resting lymphocytes (fig 3A). Sodium-dependent methionine transport in stimulated lymphocytes (72 hrs) was reduced by 56% and 76% for 30 and 90 secs, respectively, in the presence of 10 μM MTX (fig 3B). The inhibition of MTX transport was maximal at 72 hrs, was specif-

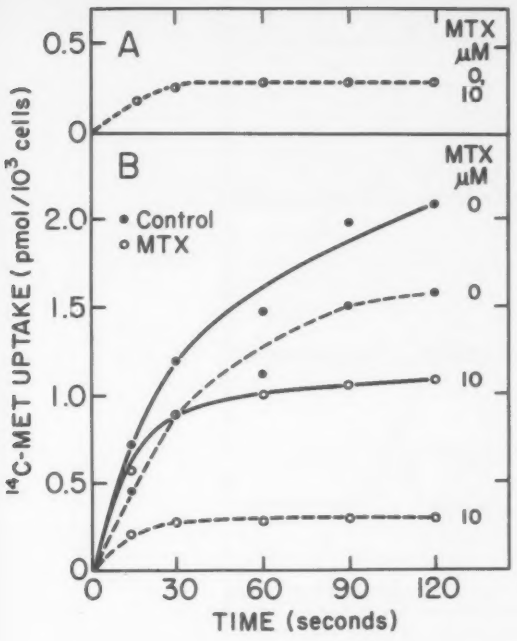

FIGURE 3.—Influence of MTX on the initial uptake of methionine in stimulated and unstimulated lymphocytes. Panel A: [^{14}C]methionine (0.5 mM) uptake was measured in unstimulated lymphocytes in the presence of sodium chloride (●) or choline chloride (○). Experiments were repeated with MTX (10 μM) added with a 2-min preincubation. Panel B: total [^{14}C]methionine (0.5 mM) uptake was measured in 72-hr stimulated lymphocytes (●—●) or total sodium-dependent methionine (○--○). The experiments were repeated (○) with MTX (10 μM) added simultaneously.

ic for sodium-dependent transport, and was only partially reversible, since washing the cells free of drug did not restore the transport of methionine. The inhibition of methionine transport by MTX was dependent on temperature (37°C was optimal) and the concentration of MTX (maximum inhibition at 25 μM). Similarly, MTX (10 μM) inhibited threonine uptake by 25% at 72 hrs (fig 5). However, MTX did not significantly alter MeAIB or BCH transport.

Amino Acid Effect on MTX Update

The uptake of MTX during the first 40 hrs following stimulation with PHA remained essentially unchanged. At 72 hrs, MTX (25 μM) uptake was nearly linear and was reduced nearly 50% by methionine (500 μM) and threonine (500 μM) (fig 6). MeAIB and BCH did not significantly block MTX uptake. Amino acids (methionine and threonine) in the presence of lower concentrations of MTX (1.0 μM) were only marginally inhibitory (data not shown).

DISCUSSION

Mitogen treatment of human lymphocytes offers a unique model for studying the activation of resting cells (1-5). Associated with this cell growth is an increase in the cellular uptake of amino acids (14-17). In the present study mitogen stimulation of lymphocytes has been characterized by several criteria. Cell volume changes as analyzed on a Coulter Counter Channelyzer and an increase in [^{3}H]thymidine incorporation into acid-precipitable material were the criteria by which normal human lymphocytes were characterized as activated. Both cell volume and DNA synthesis increased to maximum values 48-72 hrs after stimulation. Both decreased to resting cell levels with mitosis by 96 hrs.

Extensive studies defining the properties of cell growth have been reported. DNA and RNA synthesis (1-5), folate transport and metabolism (19,28,29), and amino acid transport (1-5,14-17) have been investigated in mammalian lymphocytes. The intracellular interaction of methionine with folate metabolism has been well-documented (21). Exogenous methionine and folate have been shown to be absolute requirements for DNA synthesis, which occurs 40-72 hrs after cell activation (19,29).

The uptake of four amino acids (MeAIB, AIB, BCH, and methionine) and the influence of the mitogen PHA and the folate antagonist MTX upon their uptake were studied throughout the cell cycle in the present study. The uptake of these amino acids was minimal and was primarily sodium-independent in resting lymphocytes. By 48-72 hrs after stimulation (coincident with maximum cell volume changes and DNA synthesis), increases in the transport of MeAIB, AIB, and methionine into the cell occurred and were primarily sodium-dependent.

It has previously been shown in resting lymphocytes that methionine can rapidly methylate phospholipids (30) in the resting lymphocyte. During the first 5 mins the acid-soluble labeled methionine was rapidly metabolized to another intermediate that has not been completely characterized (possibly methionine sulfoxide). This is not as evident in 72-hr stimulated lymphocytes, where the acid-soluble labeled methionine is more stable. The acid-precipitable labeled methionine in both resting and stimulated lymphocytes constitutes < 5% of the total labeled methionine transport into the cells during the first 2 mins.

MTX uptake has been well-characterized in mouse leukemic cells where the K_t is in the micromolar range (31,32). It has never been clearly demonstrated but it has been suggested that there may exist in mouse leukemia cells an additional transport pathway for MTX. This system may have a high K_t and low affinity for MTX (33). The stimulated lymphocyte system has been less well-defined for MTX transport. At low concentrations of MTX (less than micromolar) the presence of neutral amino acid has only a marginal influence on MTX uptake. However,

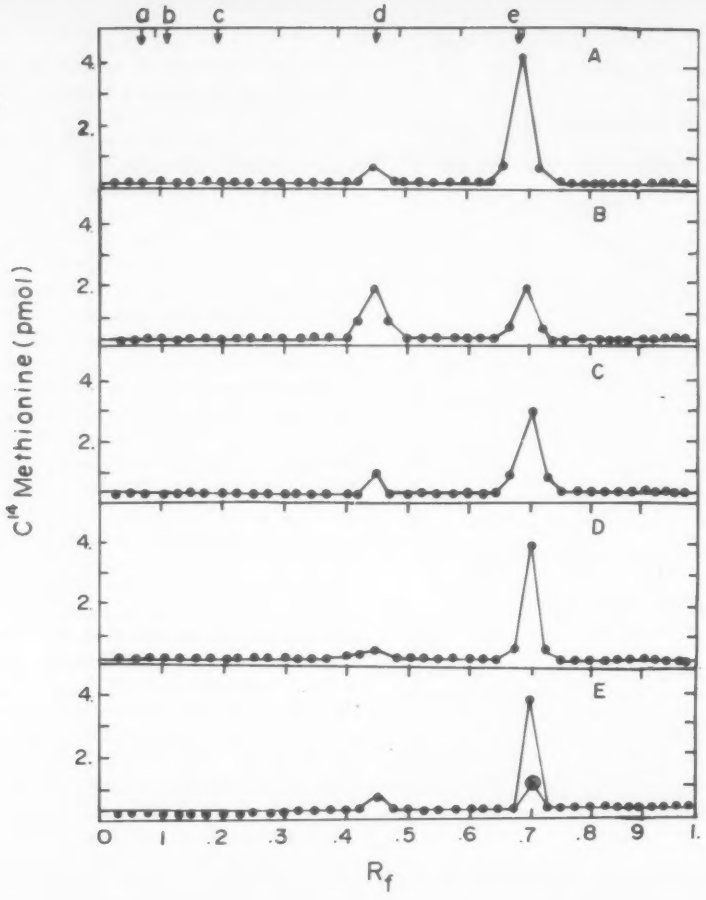

FIGURE 4.—Metabolism of labeled methionine in lymphocytes. Labeled methionine from several stages of lymphocyte activation was analyzed on descending paper chromatography. The acid-soluble fraction of the transport was assayed for labeled methionine after 5 mins. The following samples were analyzed: a standard solution of [^{14}C]methionine (panel A); resting lymphocytes (panel B); stimulated lymphocytes, 24 hrs (panel C); stimulated lymphocytes, 48 hrs (panel D); stimulated lymphocytes, 72 hrs (panel E); and methionine uptake in the presence of MTX (10 μM, ○, panel E). Unlabeled markers were co-chromatographed with labeled methionine: a = SAM; b = serine; c = SAH; d = methionine sulfoxide; and e = methionine. *See* Materials and Methods section for details on chromatography. These results were averaged from 3 experiments.

at concentrations of MTX > 10 μM these same neutral amino acids (ie, methionine and threonine) have a more substantial influence on MTX uptake (up to 40% inhibition). This is not conclusive proof for a second transport system for MTX but may suggest a partial sharing of similar transport systems at high concentrations of MTX and amino acids. The physiologic importance of this observation remains to be elucidated and is currently under investigation. It may merely reflect the conditions under which the cells are grown or a unique response of the membranes to mitogen stimulation of resting human lymphocytes.

Since stimulated human lymphocytes have a low level of dihydrofolate reductase, it has been suggested that MTX may have additional sites of action in these cells (34). Evidence presented here suggests that MTX may have an influence on the membrane. Mitogen-stimulated lymphocytes are methionine-auxotrophic (13). Recently, the methionine-auxotrophic rat hepatoma cell line HTC has been shown to have surprising substrate specificity differences for the transport of amino acid by the ASC system when compared to normal rat hepatocytes (12). In the absence of sodium, dibasic amino acids can act as substrates for the "ASC" system or as inhibitors in the

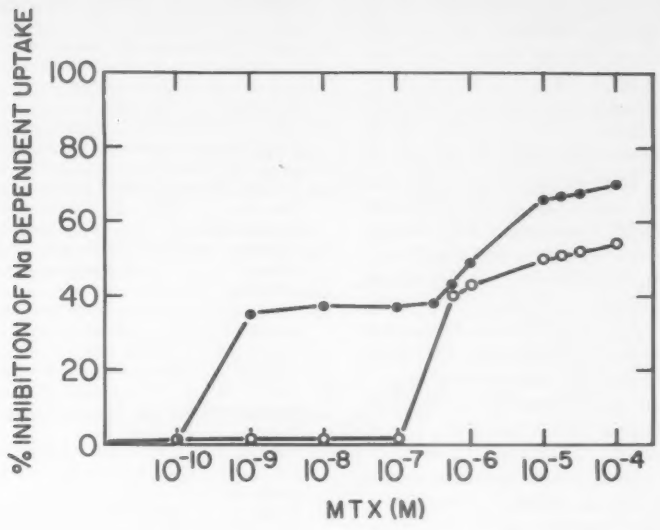

FIGURE 5.—Concentration-dependent inhibition of methionine and threonine uptake by MTX in 72-hr stimulated lymphocytes. Uptake of [^{14}C]methionine (0.5 mM, [●]) or [^{3}H]threonine (0.5 mM [○]) was measured with increasing concentrations of MTX (10^{-10} to 10^{-4} M). Control (100%) was uptake in the absence of drug. Specific activity of methionine and threonine uptake was 1.0 and 0.5 pmols/10^{3} cells/min, respectively.

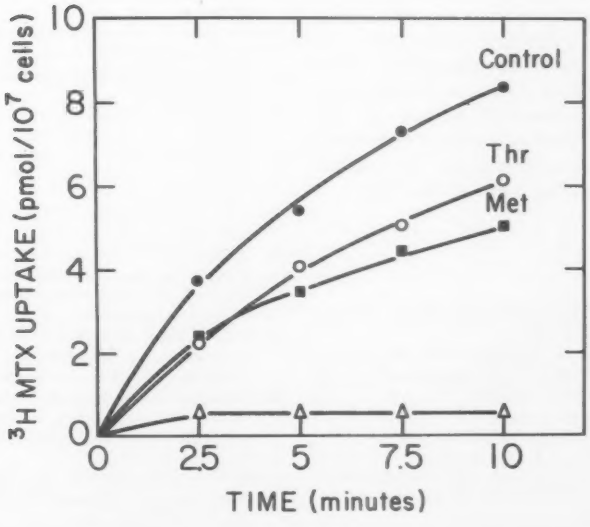

FIGURE 6.—MTX transport in lymphocytes. Influence of methionine (■) or threonine (○) on the uptake of MTX (25 μM) in 72-hr stimulated lymphocytes (●) or resting lymphocytes (△). *See* text for details.

presence of sodium (35). It has previously been shown that the 2,4-diamino pteridine diuretic, triamterene, blocks the uptake of sodium into the membrane of the tubules in the kidney (36). MTX, but not the naturally occurring folates (ie, folate and 5-methyltetrahydrofolate), can be protonated (37). Since the early work on MTX transport it has been known that a small component of labeled drug immediately binds to the cells and is not removed by extensive saline washes (32,38). For these reasons, we suspect that the MTX interaction may be with the ASC amino acid transport system, which possesses a broad specificity for substrates. However, this remains to be demonstrated conclusively. The concept that drug binding to the membrane may cause cytotoxicity is not a new concept. In L1210 cells, homofolate (39), MTX (40), cisplatin (41), and doxorubicin (42) have all been demonstrated to have membrane interactions. The L1210 cells are also methionine-auxotrophic tumor cells (20).

It has not been conclusively demonstrated that MTX binds to an amino acid carrier protein and then causes cytotoxicity. The possibilities of secondary effects such as perturbation of the Na+ gradient and/or membrane potentials must be further investigated. However, inhibition of amino acid transport by MTX directly or indirectly appears to be only partially reversible since extensive saline washes of the cells do not completely recover the transport capacity of these cells.

The results described here suggest that activation of human lymphocytes is accompanied by the stimulation of sodium-dependent methionine transport. This cell cycle-dependent increase in methionine uptake is inhibitable by MTX. Because mitogen-stimulated human lymphocytes are methionine-auxotrophic, it is suggestive that any perturbation of the methionine requirement for these cells may result in methionine deprivation. This may be an additional hypothesis for the mechanism of action of MTX in mitogen-stimulated lymphocytes and would also suggest that the nutrient transport systems of rapidly dividing cells are an additional site for possible exploitation by cancer chemotherapeutic agents (43).

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Action of Hydrolyzed Cisplatin and Some Analogs on Microtubule Protein Polymerization In Vitro[1]

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The inhibition of tubulin microtubule polymerization by cisplatin was investigated. The interaction was monitored by turbidity measurements and electron microscopy. For a 2.5 × 10^{-5} M concentration of cisplatin, the inhibition at 37°C was nearly 50% after 1 hour of incubation and 80% after 2 hours. A similar but lesser effect was observed after incubation at 4°C. One analog of cisplatin, sulfato-aqua(1,2)diamminocyclohexane platinum II, showed the same inhibition effect. [Cancer Treat Rep 67:641–646, 1983]

Cisplatin (CDDP), an uncharged square planar complex with antitumor activity, plays a major role in the chemotherapy of several human malignancies (1). Within the cell, because of the low chloride ion concentration, CDDP hydrolyzes to form mono- and di-hydroxy species (2). CDDP-DNA interactions are closely correlated with antitumor activity (2), and reactions between CDDP and intracellular proteins (in particular, protein sulfhydryl groups) have also been reported (3).

Many secondary clinical effects have been described for CDDP, including neuropathy (4,5). Moreover, Aggarval (6) described the inhibition of cytokinesis by CDDP and hypothesized that CDDP in cells induces an accumulation of Ca^{2+} responsible for the depolymerization of microfilaments. However, it is known that the polymerization of microtubules from their subunit tubulin is inhibited by organic sulfhydryl blocking agents and by some cations (7). Thus, it seemed interesting to study a possible direct interaction between tubulin and CDDP. An inhibition of tubulin polymerization might partly explain the inhibition of cytokinesis and some pharmacologic properties of this compound, in particular the neuropathic effect by axonal degeneration. We have compared this possible interaction with colchicine binding, since this alkaloid is a well-known microtubule inhibitor (8), and we have also studied the effect of some CDDP analogs, which are potential second-generation platinum drugs (9), on tubulin polymerization. Finally, we have studied the effects of platinum compounds on microtubule protein (MTP) ie, tubulin with microtubule-associated proteins, which corresponds to the physiologic conditions.

MATERIALS AND METHODS

Reagents.—CDDP of the highest purity commercially available was purchased from Roger Bellon Laboratories. CDDP analogs were supplied by Johnson Matthey Research Laboratories. MTP was purified from fresh pig brains by three cycles of in vitro assembly (37°C) and disassembly (4°C) as described previously by Shelanski et al (10), with minor modifications. Buffer A (for disassembly), pH 6.4, was: 0.1 M 2-(*N*-morpholino)-ethane sulfonic acid, 1 mM ethyleneglycol-bis(β-aminoethylether) *N*, *N'*-tetra-acetic acid, and 0.5 mM $MgCl_2$. Buffer B (for assembly), pH 6.4, was: 0.1 M 2-(*N*-morpholino)-ethane sulfonic acid, 1 mM ethyleneglycol-bis(β-aminoethylether) *N*, *N'*-tetra-acetic acid, 0.5 mM $MgCl_2$, 1 mM guanosine 5'-triphosphate, and 8 M glycerol. Protein concentration was determined according to the method of Lowry et al (11) using bovine serum albumin as standard. Colchicine was from Flucka products.

Polymerization measurements.—Tubulin assembly was monitored by turbidimetry at 335 nm with an Uvikon L.S. Printer 48 spectrometer, with a thermostated 1-cm light path cell at 37°C. The MTP concentrations were 1 mg/ml (the molarity of protein is about

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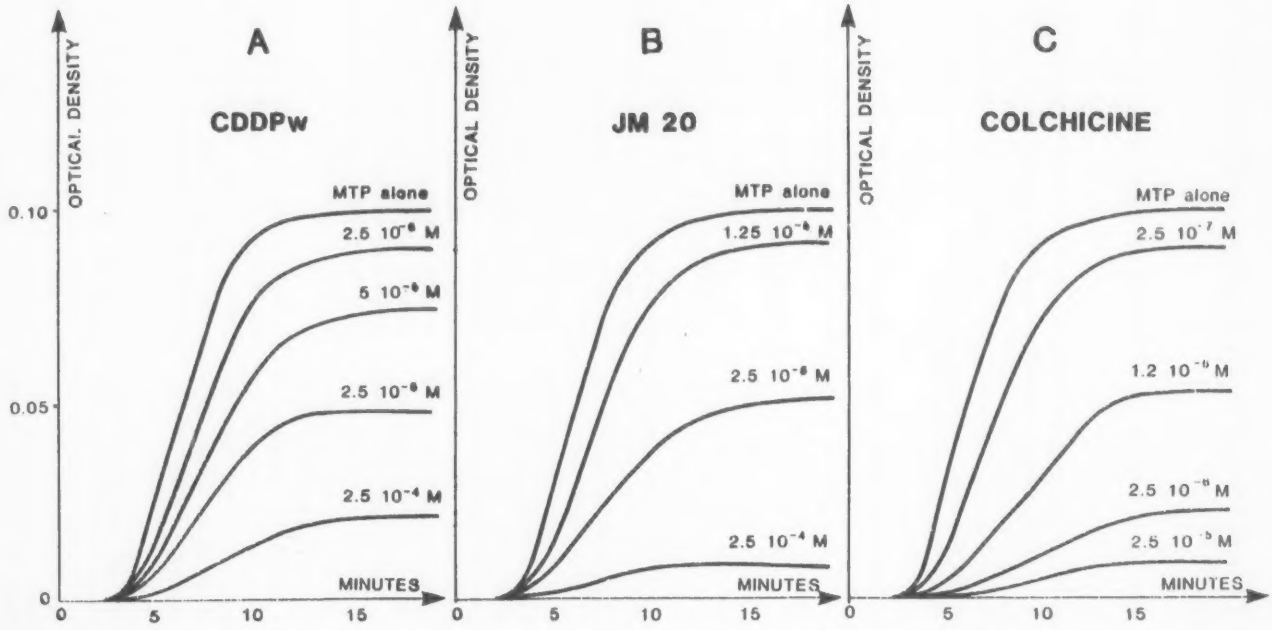


FIGURE 1.—Variation of optical density vs time during MTP polymerization. A: with CDDPw, 1-hr contact time, 37°C. B: with JM 20, 1-hr contact time, 37°C; MTP concentration, 1 mg/ml. C: with colchicine, 1-hr contact time, 37°C.

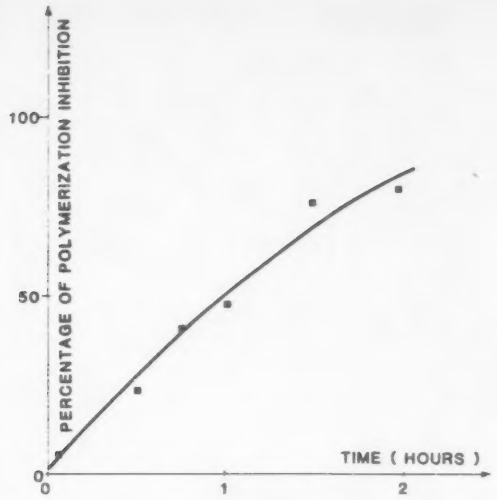


FIGURE 2.—Dependence of % inhibition vs time for a 2.5×10^{-5} M CDDPw concentration and a 1-mg/ml MTP concentration at 37°C.

10^{-5} M). The plateau value of the optical density allowed a measure of the degree of polymerization. For the determination of the inhibitory effect, 0.5 ml of hydrolyzed CDDP (12) or CDDP analogs was added to MTP solutions, in buffer A, after different contact times at 37°C or 4°C. Polymerization was then initiated by adding 1 ml of the solution to 1 ml of "reassembly" buffer (ie, buffer A with 1 mM guanosine 5′-triphosphate). In all cases, controls were run with MTP alone under the same conditions of dilution, temperature, and contact time. Polymerization in the presence of CDDP or analogs was expressed as a percentage of the control value with MTP alone. One-hour incubation at 37°C decreased the polymerization intensity of MTP alone by about 25%.

Electron microscopy.—Electron microscopy was performed with a Philips EM 400 T. Samples were taken 20 minutes after the addition of reassembly buffer and then negatively stained with 1% uranyl acetate on carbon-coated grids.

RESULTS AND DISCUSSION

Polymerization Study

No effect has been noted with nonhydrolyzed CDDP. Therefore, hydrolyzed CDDP (CDDPw) was used in all experiments. CDDP hydrolysis was checked by conductimetric measurements (12).

Figure 1 shows the action of CDDPw (fig 1A) on the MTP polymerization at 37°C after a 1-hour contact time. Fifty percent inhibition is observed with 2.5×10^{-5} M CDDPw; under the same conditions, an identical result is obtained with 1.25×10^{-6} M colchicine (fig 1C).

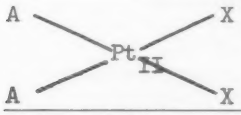
The effect of different contact times between MTP and CDDPw is shown in figure 2. For 2.5×10^{-5} M CDDPw, the percent inhibition is 80% with a 2-hour contact time as compared with 50% with the 1-hour contact time. Although the inhibition is greater after 2 hours, we have chosen the 1-hour contact time because the polymerization ability of MTP is reduced after 2 hours.

In table 1, we show the influence of temperature (37°C and 4°C) on polymerization inhibition with different CDDPw concentrations for a 1-hour contact time. CDDPw is less active at 4°C than at 37°C, yet inhibition does occur at the lower temperature. The inhibition of polymerization is nearly complete at a 2.5×10^{-4} M concentration at 37°C.

TABLE 1.—Influence of temperature on polymerization inhibition after a 1-hr contact time at different CDDPw concentrations

CDDPw concentration (M)	% polymerization inhibition at—	
	4°C	37°C
2.5×10^{-4}	45	90
2.5×10^{-5}	20	50
2.5×10^{-6}	0	9

TABLE 2.—Results obtained with CDDP analogs*



	X	A	% polymerization inhibition
JM 20	Aquasulfate	1,2-diammine cyclohexane	55
JM 8	1,1-cyclobutane dicarboxylate	Ammine	0
JM 5	2-hydroxymalonate	Ammine	0
JM 74	Malonate	1,2-diammine cyclohexane	0

* In all cases, the concentration was 2.5×10^{-5} M and the contact time was 1 hr at 37°C.

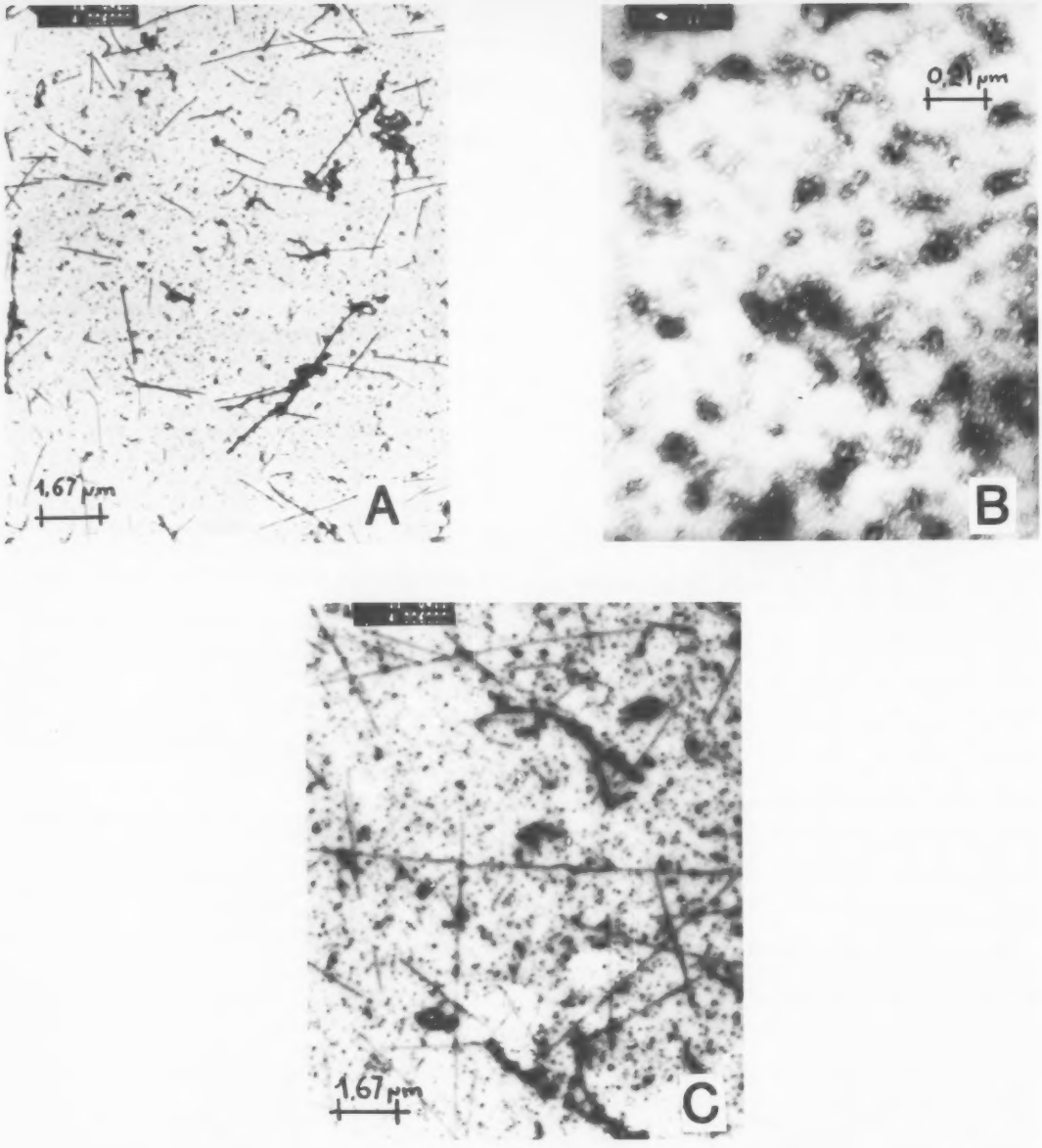


FIGURE 3.—Electron micrographs. A: MTP polymerized with 1.25×10^{-5} M CDDPw after a 1-hr contact time at 37°C (× 6000). B: MTP with 3.6×10^{-5} M CDDPw under the same conditions; no microtubular structures are identifiable (× 46,000). C: blank; MTP polymerization after a 1-hr contact time at 37°C (× 6000).

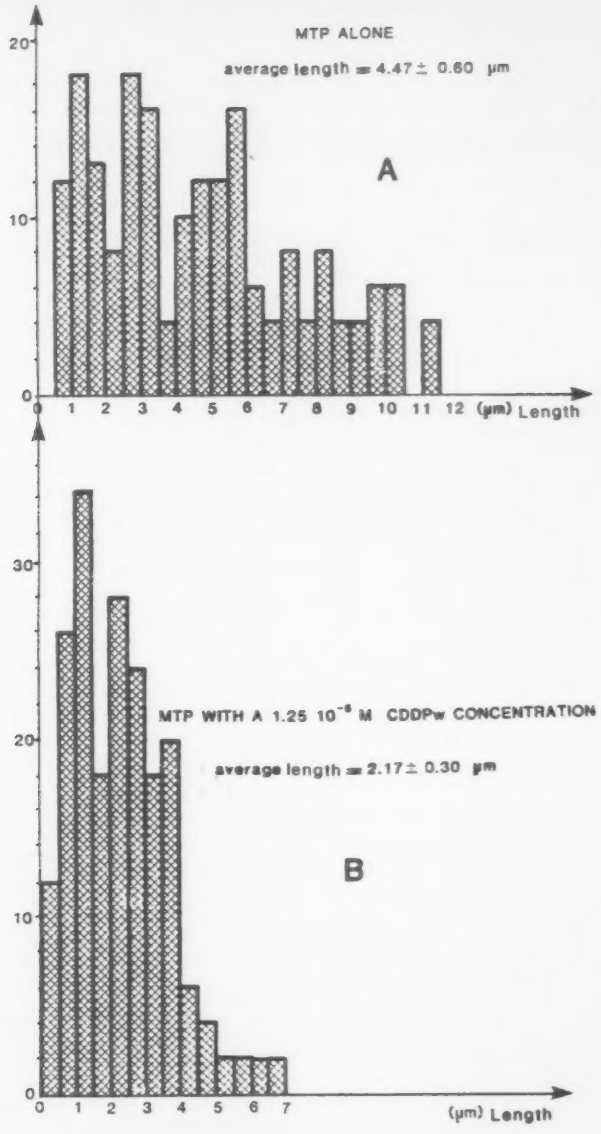


FIGURE 4.—Histograms of length frequency. Each histogram interval corresponds to 0.5 µm in length. A: MTP alone. B: MTP with 1.25×10^{-5} M CDDPw.

In table 2, we report the percent MTP polymerization inhibition by CDDP analogs (JM 20, JM 8, JM 5, and JM 74). Only JM 20 was found to influence polymerization. With a 2.5×10^{-5} M concentration of JM 20, the percent inhibition was 55%, ie, activity identical to that of CDDPw. A structure-activity relationship must be seriously considered. We can immediately observe, however, that among the CDDP derivatives, only JM 20 is a platinum compound that, like CDDP, does not present a dicarboxylate group as an X group (*see* table 2). Figure 1B represents the effect of JM 20 on MTP polymerization. This effect is nearly identical to that of CDDPw.

If the effect of CDDP on MTP polymerization is compared with the effects of colchicine and vinblastine, some similarities and some differences can be noted. After a 1-hour contact time, 10^{-6} M colchicine shows a 50% inhibition at 37°C, an inhibition which is not apparent at 4°C. Thus, at 37°C, the activity of colchicine and that of CDDPw are similar, but the effect of colchicine seems to be greater. On the contrary at 4°C, only CDDPw is active. Under similar experimental conditions for the vinca alkaloids, Himes et al (13) showed a 50% inhibition of the polymerization of beef brain tubulin at 37°C, for a 0.6×10^{-6} M inhibition concentration and a 2-minute incubation time. The immediate action of these compounds indicates that the mechanism of their binding must be different from that of CDDPw.

Electron Microscopy

Figure 3 represents electron micrographs of microtubules polymerized in the absence and in the presence of CDDPw at 1.25×10^{-5} and 3.6×10^{-5} M. Moreover, the lengths of 200 microtubules from electron micrographs were measured and data were compiled into a histogram of frequency versus length (fig 4). The electron micrographs clearly demonstrate the strong effect of CDDPw, since with 3.6×10^{-5} M the microtubules are no longer observed and with 1.25×10^{-5} M the microtubules are shortened (fig 4). The average length of microtubules in the presence of CDDPw is 2.17 μm; in the absence of CDDPw, this average length reaches 4.47 μm.

CONCLUSION

We can conclude that CDDPw reacts with MTP and inhibits the formation of microtubules. Moreover, CDDP acvitity has some similarities to that of colchicine. On the other hand, we emphasize that, in our study, Aggarval's hypothesis cannot explain the influence of CDDP on MTP polymerization, since our experiments were performed without Ca^{2+}. We cannot yet state precisely whether CDDPw reacts with tubulin or with microtubule-associated proteins and are therefore studying the interaction of CDDPw with purified tubulin (14). Whatever the nature of the interaction, the inhibition of microtubule formation may be assumed to contribute to the oncolytic and neurotropic properties of CDDP; moreover, a possible influence of CDDPw on vinca alkaloids-microtubule protein binding must be considered when combination chemotherapy with CDDP and vinca alkaloids is used.

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Dosimetry of ^{131}I-Labeled Antiferritin in Hepatoma: Specific Activities in the Tumor and Liver[1,2]

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Dosimetric studies are reported for 22 patients with hepatoma who received treatment with ^{131}I-labeled antiferritin IgG. Studies included liver and tumor volume computations based on computerized axial tomographic scan analysis, in-vivo quantitation of the activity deposited in hepatic tumors and normal liver tissue, and effective half-life measurements of the activity in the tumor, liver, and total body. Administered activities of polyclonal and affinity-column purified ^{131}I-labeled antiferritin IgG ranged from 32 to 157 mCi. Tumor volumes at the time of radioimmunoglobulin infusion ranged from 220 to 3020 cm^3 and total liver volumes ranged from 900 to 4620 cm^3. For tumor volumes ranging from 220 to 1700 cm^3, the maximum tumor activity was linearly proportional to tumor volume, but independent of antiferritin preparations and administered activities. In this range of tumor volumes, the mean value of tumor-to-liver ratios of specific activities was 4.8:1. Hepatomas ranging from 2290 to 3020 cm^3 had reduced tumor uptake of radiolabeled antiferritin IgG and had a tumor-to-liver ratio of specific activities of 1.6:1. For all patients studied there was a linear relationship between the volume of normal liver tissue and the maximum activity deposited. These data, in conjunction with toxicity studies and tumor effective half-life measurements, led to the present treatment regimen of administering 30 mCi of polyclonal antiferritin IgG on Day 0 and 20 mCi on Day 5 following the first injection. This has resulted in the same range of absorbed dose to the tumor as was achieved with larger administered activities, but with a significant reduction of total-body irradiation to the patient. [Cancer Treat Rep 67:647–658, 1983]

Phase I–II studies of ^{131}I-labeled antibody in an integrated treatment program for primary hepatic malignancies (hepatoma and cholangiocarcinoma) and dosimetric studies of ^{131}I-labeled antiferritin in hepatoma have been reported (1–3). This article reviews dosimetric data for 22 patients with hepatoma who received their first radioimmunoglobulin treatment following combination chemotherapy and external-beam liver irradiation. These data include disappearance curves for polyclonal and affinity-column purified antiferritin labeled with ^{131}I. The labeling ratio was approximately 8–10 mCi of ^{131}I/mg of IgG. Specific activities of radiolabeled antiferritin deposited in hepatic tumors and normal liver tissue, and radiation dose estimates for the tumor, normal liver, and total-body irradiation (TBI) are reported.

We have previously reported tumor localization data for patients with hepatoma who received polyclonal antiferritin with a labeling ratio of approximately 0.5 mCi of ^{131}I/mg of IgG (2). Tumor localization for these antiferritin preparations was characterized by a prolonged half-period for uptake, extending over several days, and a long effective half-life for the disappearance of isotopic IgG. Data obtained in the course of the present study showed that antiferritin preparations labeled at 8–10 mCi/mg resulted in maximum tumor localization in < 1 hour following administration. Furthermore, disappearance characteristics and effective

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half-lives were strongly affected by increases in the labeling ratio and affinity-column chromatography. However, the range of specific activities deposited in the liver and tumor was the same for all antiferritin preparations studied.

An analysis of dosimetric data showed that for tumor volumes ranging from 220 to 1700 cm^3 (18 of 22 patients) the maximum activity deposited was linearly proportional to tumor volume. The linear relationship between tumor volumes and the maximum activity deposited in the tumor implied that large administered activities did not result in increased tumor deposition of radiolabeled antiferritin. This consideration, in conjunction with an evaluation of the toxicity associated with the administration of isotopic IgG (3) and tumor effective half-life measurements, led to the present treatment regimen of administering 30 mCi of polyclonal antiferritin on Day 0 and 20 mCi on Day 5 following the first injection. Tumor localization data have demonstrated that the two injections resulted in the same initial dose rates to the tumor and that these dose rates were of the same magnitude as were achieved with larger administered activities. The current regimen at the same time has resulted in a reduction of TBI for the patient and reduced radiation exposure for personnel.

This article is concerned primarily with dosimetric data and conclusions based on them. Results for the therapeutic efficacy of the integrated treatment program for primary hepatic malignancies will be reported separately.

MATERIALS AND METHODS

Ferritin Purification

Tumor ferritin was purified and crystallized from spleens involved with Hodgkin's disease as previously described (4). A minimum of 1 kg of tissue was processed at any one time to minimize variation from one batch to the next. Hodgkin's tumor ferritin preparations to date have been immunologically identical as determined by immunodiffusion and radioimmunoassay.

Production of Antisera

New Zealand white rabbits and pigs maintained at the Hopkins farm were immunized intradermally with 100–200 μg of crystallized Hodgkin's ferritin emulsified in complete Freund's adjuvant. After booster injections with ferritin, 50 ml of blood was removed from each rabbit by sterile cardiac puncture, allowed to clot at room temperature for 1 hour, and refrigerated at 4°C ovenight for clot retraction. Pigs were placed in a restraint chute and bled percutaneously via the anterior vena cava and handled as above.

Preparation of IgG

Three preparative procedures were compared for the production of IgG. A minimum of 1 L of antisera was processed at a time to minimize variations in preparations.

IgG was initially prepared by two precipitations with ammonium sulfate at 33% saturation. The final IgG preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.6, plus 0.15 M sodium chloride (phosphate-buffered saline [PBS]) and dialyzed against the same buffer overnight.

Present IgG preparations (3 years) were prepared by ion-exchange chromatography with DEAE cellulose (DE-52, Whatman, Ltd, England). The antisera were dialyzed against 0.01 M sodium phosphate buffer, pH 8.0, and incubated with DE-52 equilibrated with the same buffer at a ratio of 1 ml of serum/5 g of cellulose for 1 hour at 4°C. The IgG was removed by suctioning through a sterile Buchner funnel, concentrated by ammonium sulfate precipitation, and dialyzed against PBS overnight.

Affinity-column purified IgG was prepared as described by Eveleigh (5). Purified Hodgkin's ferritin was covalently linked to CnBr-activated Sepharose 4B as described by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ). Adsorption and elution were performed using an automated system (5,6). The eluted antibody was immediately dialyzed against PBS and concentrated by ammonium sulfate precipitation.

All samples were sterile filtered and tested for bacterial contamination and pyrogenicity prior to freezing.

Radiolabeling

The IgG samples were radiolabeled with ^{131}I by the lactoperoxidase method (7). The labeled IgG was separated from unbound label using a Pharmacia PD-10, G-25 column (Pharmacia Fine Chemicals).

Liquid lactoperoxidase has been replaced by commercially prepared lactoperoxidase glucose-oxidase beads (Bio-Rad Laboratories, Richmond, CA). Radiolabeled IgG was separated from free iodine as described above. Sterility and pyrogen testing of all samples was performed prior to administration to patients.

Specific activities of early samples were approximately 0.5 mCi/mg of IgG, and have since been increased to approximately 8–10 mCi/mg of IgG. Affinity-column purified IgG was labeled at the higher specific activity. The labeled IgG was used either undiluted, diluted with specific IgG, diluted with normal IgG of the same species, or diluted with 1% normal human serum albumin.

Tumor and Liver Volume Computations

Volume computations were based on the analysis of patients' computerized axial tomographic (CAT) scans

obtained 1–7 days prior to the administration of radiolabeled antiferritin. The liver was scanned at contiguous 1-cm intervals using a slice thickness of 1 cm, and normal and tumor-bearing regions of the liver were contoured manually in each CAT scan slice. Following each patient's CAT scan, a calibration phantom was imaged employing the same scanner settings as used for the patient. The contours in the entire sequence of each patient's CAT scans and the calibration data were digitized as previously described (2). For volume computations, the surface areas in each CAT scan slice were obtained by numerically integrating around the closed contours representing normal and tumor-bearing regions of the liver. The surface areas were multiplied by the slice thickness to obtain the volume for each slice, and liver and tumor volumes were computed by addition of the slice volumes. Furthermore, the computer software permitted reconstruction of anteroposterior views of the tumor and liver at any level.

In Vivo Quantitation of Radiolabeled Antiferritin in the Tumor and Liver

The ^{131}I activity in the tumor and liver was quantitated by the method of conjugate gamma camera views (8,9). Two to four days prior to the infusion of radiolabeled antiferritin, anterior and posterior gamma camera images of the liver were obtained using 3 mCi of ^{99m}Tc sulfur colloid injected iv. These images served to identify normal and tumor-bearing regions of the liver in radionuclide scans and to position patients under the gamma camera for optimum views of the liver. In addition, sulfur colloid images were utilized to put ink marks on patients' skins for subsequent bedside monitoring of ^{131}I liver and tumor count rates. Following the ^{99m}Tc sulfur colloid images, transmission measurements were made for each patient using a flood source that contained about 2 mCi of ^{131}I. The flood source was imaged with and without the patient between source and gamma camera, and transmission factors for the tumor and liver were determined from these images using a regions-of-interest program. A large-field of-view camera equipped with a 400-keV collimator was employed for the 364-keV emissions of ^{131}I and a 20% energy window selected to reduce scattered radiation effects. The same collimator and energy window were used for the 140-keV emissions of ^{99m}Tc sulfur colloid. Images were recorded and analyzed using a minicomputer interfaced with the gamma camera. The computer permitted definitions of regions of interest and provided anterior and posterior count-rate information for each region.

Two to eight days after administration of ^{131}I-labeled antiferritin, patients were reinjected with 3 mCi of ^{99m}Tc sulfur colloid, and anterior and posterior ^{99m}Tc and ^{131}I images were obtained. In this case, however, a 10% window was selected for the Tc images to reduce the effect of Compton scattered ^{131}I photons. The second Tc injection eliminated the problem of patient movement between scans and facilitated definitions of regions of interest corresponding to tumor-bearing and normal liver regions in ^{131}I images. A minimum of 500,000 counts was accumulated for all images, and the activity of ^{131}I-labeled antiferritin deposited in the tumor and liver was calculated in the same manner as described previously (2). The system's calibration factor was determined from a source of known activity, measured in a well-type ionization chamber whose calibration was traceable to the National Bureau of Standards. The activity calculations were based on the system's calibration factor, the transmission factors for tumor and liver measured for every patient, anterior and posterior count-rate information obtained for every patient using a regions-of-interest program, and a correction factor which compensates for self-attenuation of the radiation within the source (2,8,9).

Figure 1 demonstrates this method of imaging and shows anterior ^{99m}Tc sulfur colloid and ^{131}I images obtained 4 days after injection of 50 mCi of radiolabeled antiferritin. The corresponding computer-generated view shows the equivalence of tumor-bearing and normal liver regions in radionuclide images and volume computations based on CAT-scan analysis.

Effective Half-Life Measurements

Radiolabeled IgG was administered under close medical observation for 2 hours, and then patients were observed more routinely. Half-life measurements, therefore, started about 2 hours after administration and were continued for the duration of the patients' hospitalization. The effective half-life of radiolabeled antiferritin for total-body activity was determined by monitoring the exposure rate at the bedside with a survey meter at a distance of ≥ 1 m from the patient. Relative measurements of the disappearance of isotopic immunoglobulin in the tumor and liver were made with an NaI (T1) scintillation probe utilizing skin ink marks in the same manner as previously described (2). It is emphasized that the measurements were *relative* ones, made for the purpose of determining effective half-lives for total-body activity and activity in the tumor and liver. Consistency checks of survey meter readings, used for exposure-rate measurements, and the scintillation probe were made using standards of known activity. Additionally, relative measurements were verified by sequential gamma camera imaging, as shown in the Results section. It has been our experience in the previous (2) and in the current studies that half-lives based on bedside measurements agreed within 3%, with half-lives based on gamma camera scans. Bedside measurements have the advantage in that they can be made

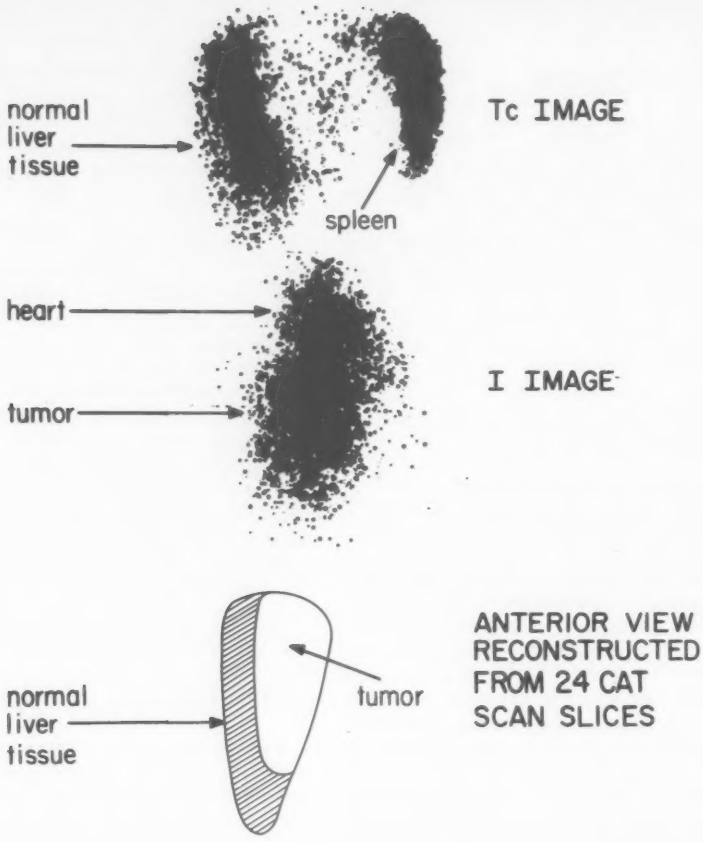

FIGURE 1.—Anterior ^{99m}Tc sulfur colloid (Tc) and ^{131}I (I) gamma camera images of a patient with hepatoma obtained 4 days after injection of 50 mCi of ^{131}I-labeled antiferritin. The Tc image shows a large defect in the left lobe of the liver. The I image demonstrates the cardiac silhouette due to radiolabeled antibody in the blood circulation and antiferritin deposition in the tumor. The tumor-to-liver ratio of specific activities for this patient was 3.7:1. The computer-generated anterior view, reconstructed from a sequence of 24 CAT-scan slices, demonstrates the equivalence of normal and tumor-bearing regions of the liver in radionuclide images and volume computations based on CAT-scan analysis.

frequently, accurately, and without undue radiation exposure of personnel.

RESULTS

Disappearance Curves

Radiolabeled porcine and rabbit-derived antiferritin IgG were equivalent in terms of the activity deposited in the tumor, tumor localization kinetics, and TBI. Antiferritin preparations derived from both species are, therefore, referred to as polyclonal antiferritin IgG.

Four of the 22 patients were administered polyclonal antiferritin IgG with a labeling ratio of 0.5 mCi of ^{131}I/mg of IgG. Dosimetric studies for these four patients have been reported in a previous publication (2). The remaining 18 patients were infused with affinity-column purified or polyclonal antiferritin labeled at 8-10 mCi of ^{131}I/mg of IgG. The higher labeling ratio was used because of its theoretic potential to deliver a greater radiation dose to the tumor. Furthermore, it significantly reduced radiation exposure to personnel during the labeling procedures because of the smaller volume of IgG that needed to be labeled. Representative disappearance curves for these antiferritin preparations are presented below, and results for all 22 patients are summarized in table 1.

TABLE 1.—Administered activities, antiferritin preparations, labeling ratios, and effective half-lives

No. of patients	Administered activity (mCi)	Antiferritin preparation	Labeling ratio (mCi/mg)	Effective half-life (days) Tumor and liver Mean	Tumor and liver Range	Body Mean	Body Range
4*	93–157	Polyclonal	0.5	7.4	6.8–7.8	3.6	2.9–4.3
3	84–153	Affinity-purified	8–10	0.52†	0.25–0.23	0.30	0.14–0.48
				3.0‡	1.6–3.4	2.2	1.4–2.5
5	37–117	Polyclonal	8–10	2.9	1.9–4.2	2.7	1.4–3.6
10§	32–100	Polyclonal diluted with unlabeled protein	8–10	4.6	3.9–5.4	3.5	3.3–4.2

*Tumor localization kinetics for these patients were also characterized by prolonged half-periods for uptake (ref 2).
†Effective half-life for fast disappearance phase of affinity-purified antiferritin.
‡Effective half-life for slow disappearance phase of affinity-purified antiferritin.
§Includes 7 patients who were administered 30 mCi on Day 0 and 20 mCi on Day 5 for a total of 50 mCi.

Figure 2 shows disappearance curves for total-body activity and activity in the tumor and liver for affinity-column purified antiferritin. These disappearance curves were resolved into sums of two exponential terms using standard techniques (10,11). The two exponential terms correspond to a fast disappearance phase with a short effective half-life (T_e) at early times after injection, followed by a slower disappearance phase with a longer effective half-life at later times. Furthermore, liver and tumor count rates in this figure demonstrate that about 70% of the initial activity deposited in these tissues disappears within 2 days after injection.

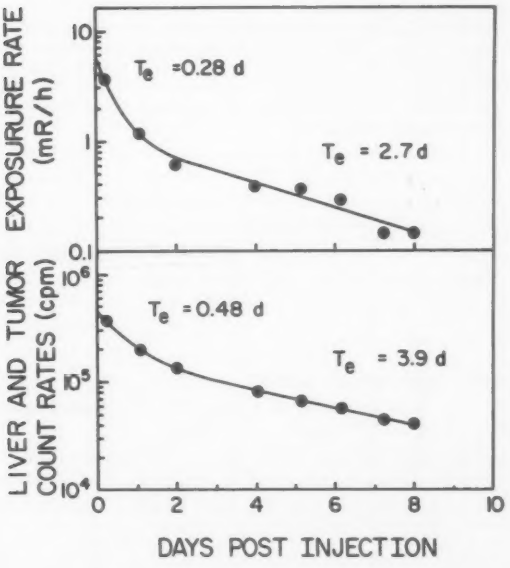

FIGURE 2.—Representative disappearance curves for affinity-purified antiferritin with a labeling ratio of 10 mCi/mg. The upper and lower graphs show the disappearance of total-body activity and activity in the liver and tumor, respectively. Both curves were resolved into sums of exponential terms corresponding to short effective half-lives (T_e = 0.28 days and 0.48 days) at early times after injection and longer effective half-lives (T_e = 2.7 days and 3.9 days) at later times. About 70% of the initial activity in the liver and tumor disappeared within 2 days after injection. The ratios of fast-to-slow disappearance terms at t = 0 were 4:1 and 1.7:1 for total-body activity and activity in the tumor and liver, respectively.

Disappearance curves for polyclonal antiferritin with a labeling ratio of approximately 8–10 mCi of ^{131}I/mg of IgG are shown in figure 3. These curves were characterized by single exponential terms and short effective half-lives, but without the early disappearance phase and rapid loss of activity observed for affinity-column purified antiferritin.

Figure 4 shows representative disappearance curves with the same labeling ratio, but diluted with unlabeled protein immediately after the labeling procedure so that the total volume was increased fivefold. The amount of unlabeled protein added was adjusted so that after dilution, the protein content of the preparations was 2 mg of specific IgG or normal IgG of the same species per millicurie of ^{131}I. For normal human serum albumin the amount of unlabeled protein added was 3 mg/mCi of ^{131}I. These curves were also characterized by single exponential components but demonstrated longer effective half-lives than were obtained with either affinity-column purified or undiluted polyclonal antiferritin. Furthermore, the data in figure 4 were representative of the present treatment regimen for the administration of radiolabeled antiferritin to patients with hepatoma. The administered activities were 30 mCi on Day 0 and 20 mCi on Day 5 following the first injection. Liver and tumor count rates demonstrated that the first and second injections resulted in the same initial activities. These results were verified by sequential gamma camera scans and in vivo quantitation (fig 5).

Table 1 shows the number of patients for each antiferritin preparation, the range of administered activi-

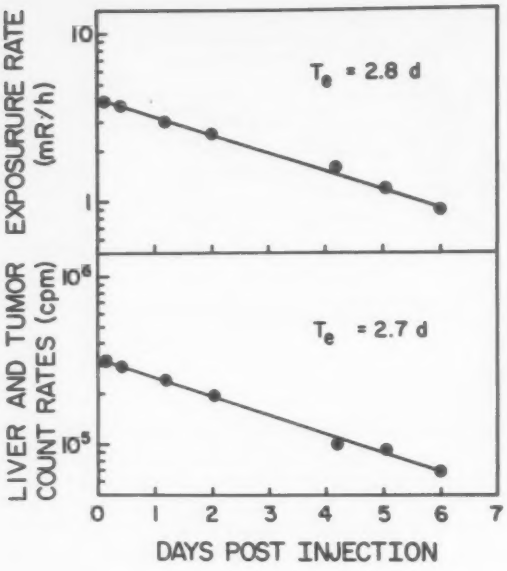

FIGURE 3.—Representative disappearance curves for polyclonal antiferritin with a labeling ratio of 10 mCi/mg. The curves correspond to single exponential terms.

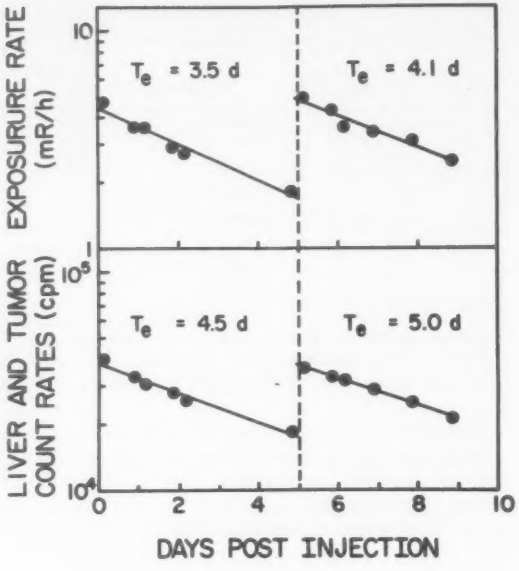

FIGURE 4.—Representative disappearance curves for polyclonal antiferritin with a labeling ratio of 10 mCi/mg and diluted with unlabeled protein. The administered activities were 30 mCi on Day 0 and 20 mCi on Day 5, respectively. The curves were characterized by single exponential terms and initial liver and tumor count rates were the same for both injections.

ties, and the range and mean value of the effective half-life for the tumor and liver and total-body activity. For affinity-column purified antiferritin, the effective half-life for both the fast and slow disappearance phase is given. Ten of the 22 patients were administered polyclonal antiferritin diluted with unlabeled protein. The data show that diluted polyclonal antiferritin resulted in a longer effective half-life than either affinity-column purified or undiluted polyclonal antiferritin. All of the unlabeled proteins used for dilution (specific IgG, normal IgG, and 1% normal human serum albumin) led to a longer effective half-life within the same range and had no effect on tumor uptake of radiolabeled IgG.

Specific Activities in the Tumor and Liver

Calculations of specific activities were based on the assumption of uniform distribution of these activities in each target. Specifically,

$$C = A/m \qquad \mu Ci/g \qquad [1],$$

where A is the maximum value of the activity in units of microcuries deposited in the tumor and normal liver, respectively, m denotes the corresponding mass in grams, and C is the specific activity in units of microcuries of ^{131}I per gram of tissue. Maximum activity values were chosen as a basis for comparison to allow for the greatly differ-

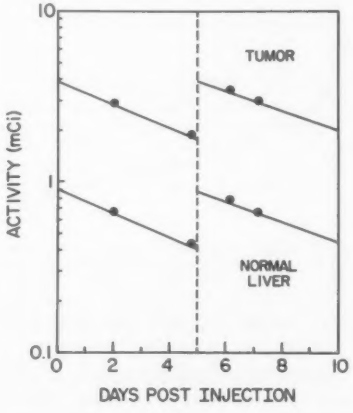

FIGURE 5.—Results of sequential gamma camera scans and in vivo quantitation of the activity in the tumor and normal liver for the same patient as in fig 4. Circles represent the results of in vivo quantitation; lines correspond to the effective half-life measurements given in fig 4. Data demonstrate that the 2 administrations of radiolabeled antiferritin deposit the same initial activities but at different levels of saturation in tumor and liver tissues.

ing tumor localization kinetics and disappearance curves of the antiferritin preparations listed in table 1. For the four patients who were administered antiferritin with a labeling ratio of 0.5 mCi/mg, maximum values of the activity were determined from the maxima in the activity-versus-time curves reported previously (2). For all other patients maximum activity values were obtained by extrapolating the disappearance curves for the tumor and liver to t = 0 (figs 2–4). Unit density was used to convert liver and tumor volumes computed from CAT scans to mass in grams.

The results of these computations are summarized in table 2. Eighteen patients had tumor volumes ranging from 220 to 1700 cm^3, and for these patients the mean value of the specific activities in the tumor was 8.4 μCi/g, with a tumor-to-liver ratio of 4.8:1. Four patients had tumor volumes ranging from 2290 to 3020 cm^3; for these patients the mean value of specific activities in the tumor was 2.4 μCi/g, with a tumor-to-liver ratio of 1.6:1. Furthermore, the range of specific activities for large tumor volumes was distinctly lower than for the smaller volumes, whereas the specific activity in normal liver tissue was nearly the same for all patients. This is graphically shown by the histograms in figure 6.

The estimated uncertainties in the specific activity values for tumor and normal tissues, based on the accuracy and reproducibility of volume computations and in vivo quantification, were approximately 15%–20%. There were no quantifiable nonuniformities, by external gamma camera imaging, of the antiferritin deposition in tumors and normal liver, respectively. This justified the assumption of uniform distribution on the *macroscopic level* and implied that the errors associated with this assumption were too small to significantly affect activity calculations per gram of tissue. We recognize, however, that gamma camera imaging is insensitive to nonuniformities in activity distributions which may occur on the cellular level.

Linear Relationships Between Tumor and Liver Activities and Tumor and Liver Volumes

Figure 7 shows a plot of tumor volume versus maximum activity deposited in the tumor, ranging from approximately 2 to 16 mCi. The line was the result of a linear regression analysis of the form $y = a + bx$, where a and b were the intercept and slope, respectively.

For the 18 patients with tumor volumes of ≤ 1700 cm^3, the equation of regression was

$$A_T = 1.14 + 6.89 \times 10^{-3} V_T \text{ mCi} \quad [2],$$

where V_T was the tumor volume in cm^3 and A_T was the activity in millicuries. The correlation coefficient between the data for these 18 patients and the regression equation was 0.88 ($P < 0.001$). The standard deviation of the mean was 1.45, of the intercept (1.14) was 0.86, and of the slope (6.89×10^{-3}) was 0.89×10^{-3}.

For comparison, the activities for tumor volumes of ≥ 2290 cm^3 are shown in the same figure. It was evident that for these large tumors antiferritin deposition was lower in total and specific activity than for the smaller tumors.

The activity deposited in normal liver tissue versus normal liver volume is shown in figure 8. The line represents the linear regression equation

$$A_L = 0.410 + 1.40 \times 10^{-3} V_L \text{ mCi} \quad [3],$$

where V_L was the normal liver volume in cm^3 and A_L was the normal liver activity in millicuries. The correlation coefficient for all 22 patients was 0.89 ($P < 0.001$). The standard deviation of the mean was 0.542, of the intercept (0.410) was 0.256, and of the slope (1.41×10^{-3}) was 0.15×10^{-3}.

The ratio of the slopes in Equations 2 and 3 was equal to 4.88, in agreement with the tumor-to-liver ratio given in table 1 for tumor volumes ranging from 220 to 1700 cm^3.

The linear relationship between tumor volume and the activity deposited in the tumor meant that large administered activities did not result in increased tumor deposition of radiolabeled antiferritin. This is shown graphically in figure 9 which is a plot of administered activities versus the percentage of these activities taken up in the tumor. The fraction deposited in the tumor ranged from approximately 3% for the largest administered activities to approximately 30% for lower administered activities. The variation in tumor uptake at each level of administered activity corresponded to variations in tumor volumes.

TABLE 2.—Specific activities (μCi/g) in tumor and liver tissues and tumor-to-liver ratios

No. of patients	Tumor volumes (cm^3)	Mean (range)— Specific activity		Tumor-to-liver ratios
		Tumor	Liver	
18	220–1700	8.4 (6.2–12)	1.8 (1.1–3.4)	4.8 (1.9–7.0)
4	2290–3020	2.6 (1.7–3.6)	1.6 (1.1–2.3)	1.6 (1.1–2.2)

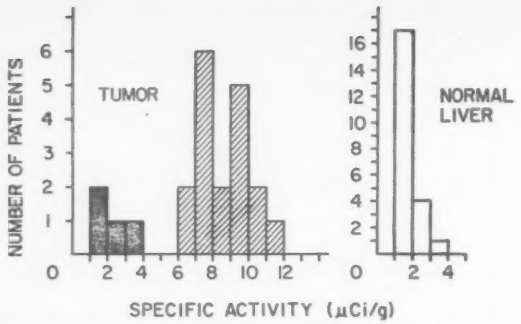

FIGURE 6.—Distribution of specific activities in tumor and liver tissues, plotted in unit intervals, for 22 patients with hepatoma. Cross-hatched bars represent 18 patients with tumor volumes ranging from 220 to 1700 cm^3 and shaded bars correspond to 4 patients with tumor volumes ranging from 2290 to 3020 cm^3. The specific activity for large tumor volumes was distinctly lower than for smaller tumor volumes. Specific activities in normal liver tissue were approximately the same for all 22 patients.

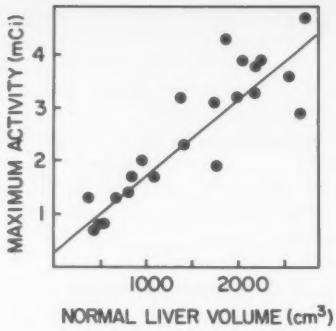

FIGURE 8.—Volume of normal liver tissue vs maximum activity for 22 patients with hepatoma. The line is the result of a linear regression analysis. The correlation coefficient between the data points and the regression equation for all 22 patients is 0.89 ($P < 0.001$).

Radiation Dose Estimates

Radiation dose calculations, based on the assumption of uniform distribution of activities, were made according to the Medical Internal Radiation Dose Committee of the Society of Nuclear Medicine method of absorbed fractions (12,13). Formulas for the application of this method to the dosimetry of ^{131}I-labeled antiferritin in patients with hepatoma have been presented in a previous publication (2). The same method of calculation was used in the current study.

Input parameters for dose calculations were the administered activities, patients' weights, effective half-lives for the tumor, liver, and total body, liver and tumor volumes, and the activities deposited in these tissues. Physical constants, ie, mean energy per unit cumulated activity (equilibrium dose constants) and specific absorbed fractions for the penetrating radiations of ^{131}I were obtained from available tables (14,15). The results are summarized in table 3. For completeness, the range of administered activities and antiferritin preparations is also listed. The range of maximum dose rates for the tumor and liver was approximately the same for all antiferritin preparations. This was a direct consequence of the fact that the range of specific activities in the tumor and liver was the same for all preparations. The lowest dose rates (1.1–1.8 rads/hour) corresponded to patients with the largest tumors (2290–3020 cm^3).

The variation in the radiation dose to the tumor and liver arose from the different tumor localization kinetics and disappearance characteristics of the antiferritin preparations. Affinity-column purified antiferritin deliv-

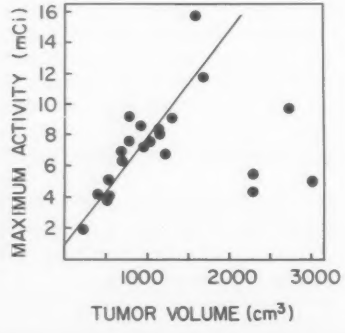

FIGURE 7.—Tumor volume vs maximum activity deposited in the tumors of 22 patients with hepatoma. The line represents the result of a linear regression analysis for tumor volumes ranging from 220 to 1700 cm^3 (18 patients). The correlation coefficient between data points and regression equation is 0.88 ($P < 0.001$). Data for large tumor volumes (2290–3020 cm^3) demonstrate reduced antiferritin uptake.

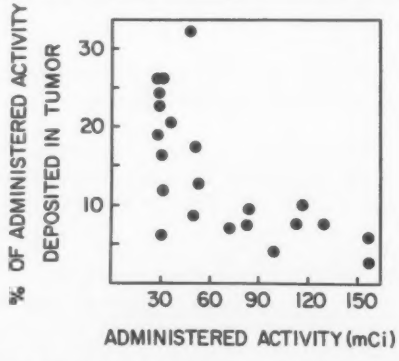

FIGURE 9.—% of the administered activities deposited in the tumor demonstrate that large administered activities do not result in increased tumor deposition. Tumor uptake ranges from approximately 3% at 157 mCi to 30% in the lower range of administered activities. The variation in tumor deposition at each level of administered activity is due to differences in tumor volumes.

TABLE 3.—Administered activities, antiferritin preparations, dose rates, and doses

No. of patients	Administered activity (mCi)	Antiferritin preparation	Range of—					
			Maximum dose rate (rads/hr)			Dose (rads)		
			Tumor	Liver	TBI	Tumor	Liver	TBI
4*	93-157	Polyclonal	3.6-5.6	1.0-2.8	1.0-1.9	1500-2200	400-1000	110-220
3	84-153	Affinity-purified	1.8-5.9	1.6-2.7	0.85-1.7	200-250	80-160	20-50
5	37-117	Polyclonal	3.1-4.2	0.48-1.3	0.25-1.1	360-610	50-150	25-130
3	32-100	Polyclonal diluted with unlabeled protein	1.1-5.1	0.50-1.0	0.30-0.63	200-780	90-120	40-75
7	30/20†	Polyclonal diluted with unlabeled protein	1.5-5.7	0.65-1.1	0.25-0.35	300-1300	150-300	30-50

* Labeling ratio for these patients was 0.5 mCi of ^{131}I/mg of IgG; for all other patients it was 8-10 mCi/mg.
†30 mCi on Day 0 and 20 mCi on Day 5. Maximum dose rates were the same for the 2 injections.

ered the lowest radiation dose because of the short effective half-life and loss of activity during the early disappearance phase. Similarly, polyclonal antiferritin with a labeling ratio of 8–10 mCi/mg resulted in a low radiation dose because of the short effective half-life. Polyclonal antiferritin with the same labeling ratio, but diluted with unlabeled protein, had a longer effective half-life and consequently delivered a higher radiation dose. The results for seven patients who were administered 30 mCi on Day 0 and 20 mCi on Day 5 demonstrated that this treatment regimen resulted in a tumor dose comparable to that achieved with higher administered activities and at the same time had reduced TBI. The lowest dose rate and dose in this group corresponded to one patient with a tumor volume of 3020 cm^3.

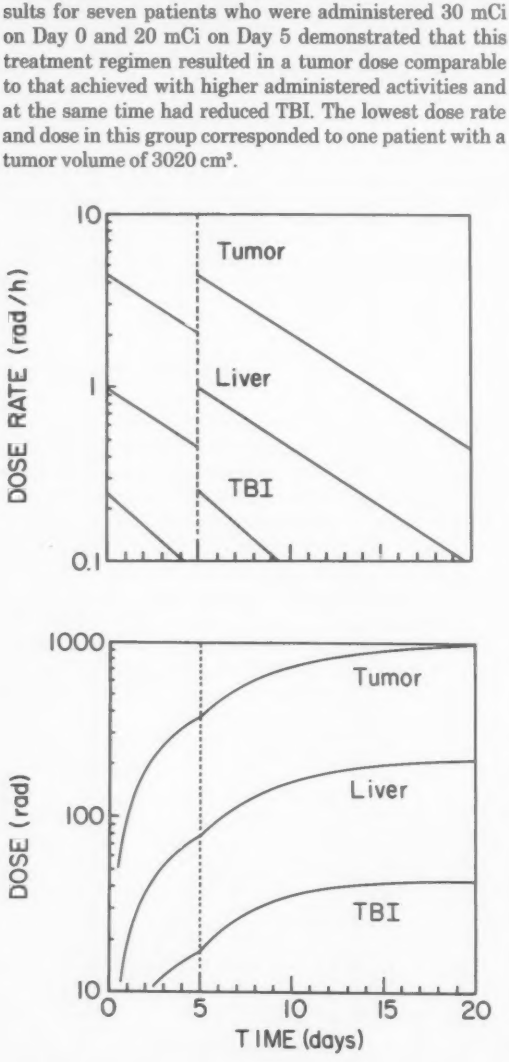

FIGURE 10.—Calculated dose rates and dose as a function of time after injection of 30 mCi on Day 0 and 20 mCi on Day 5. Curves were generated as described in the text. For complete disappearance of the isotope the dose was approximately 1100 rads to the tumor, 230 rads to normal liver tissue, and 45 rads for TBI.

Representative calculations of the dose and dose rate for the tumor, normal liver tissue, and TBI as a function of time after injection of 30 mCi on Day 0 and 20 mCi on Day 5 are shown in figure 10. These curves were generated by using as input parameters a body weight of 70 kg, mean values of effective half-lives for polyclonal antiferritin diluted with unlabeled protein (4.6 days for the tumor and liver; 3.5 days for TBI) given in table 1, and mean values of specific activities (8.4 μCi/g for the tumor; 1.8 μCi/g for normal liver tissue) given in table 2. The resulting initial mean dose rates were approximately 4.4, 1.0, and 0.25 rads/hour for tumor, normal liver tissue, and TBI, respectively. For complete disappearance of the isotope this corresponded to a mean dose of approximately 1100 rads to the tumor, 230 rads to normal liver tissue, and 45 rads of TBI.

The estimated error in calculations of the mean dose for the tumor and liver was approximately 25%–30% (2). Dose calculations should, therefore, be taken as being indicative of the magnitude of dose rates (and dose) that had been achieved rather than exact numbers. The error estimate was based on phantom and patient studies (8,9) which indicated that the method of in vivo quantitation applied in the current study was accurate to approximately 10%, reproducibility and accuracy of tumor and liver volume computations of about 10%, a 5% uncertainty in effective half-life measurements, and the fact that most of the radiation dose for the tumor and liver was provided by the nonpenetrating radiations of ^{131}I (2). The error estimate for liver and tumor volume computations was consistent with error determinations reported elsewhere for volume calculations based on CAT-scan analysis (16,17).

DISCUSSION

Results obtained in this study have shown that the range of specific activities deposited in the liver and tumor was the same for all antiferritin preparations administered. However, tumor localization kinetics and effective half-lives were strongly influenced by affinity chromatography purification, increases in the labeling ratio, and subsequent dilution with unlabeled protein.

The disappearance curves for affinity-column purified antiferritin were characterized by two exponential components corresponding to a short half-life at early times after injection with rapid loss of activity and a longer effective half-life at later times. The two disappearance phases suggest that the procedures used in affinity-chromatography purification may have damaged the IgG although in vitro specificity was retained. Furthermore, blood samples were obtained daily from patients who were administered either polyclonal or affinity-column purified radiolabeled IgG. In all cases, ^{131}I was > 95% precipitable with 10% trichloroacetic acid, indicating that in the blood the isotope was bound to protein. Column

chromatography and immunoprecipitation showed that ^{131}I was bound to the injected antiferritin IgG. There was no evidence for dehalogenation, even in those blood samples which were obtained at early times after injection from patients who were administered affinity-column purified antiferritin IgG. Consequently, the rapid loss of activity at early times after injection of affinity-column purified preparations could not be ascribed to dehalogenation. The high specific activity of the labeled product caused additional radiation-induced damage. This interpretation was consistent with the short effective half-life of polyclonal antiferritin labeled at the same ratio. The effective half-life associated with the second disappearance component of affinity-column purified antiferritin was nearly identical with the effective half-life of undiluted polyclonal antiferritin (table 1). Affinity-column purified and undiluted polyclonal antiferritin had specific activities of 10 mCi/ml and was stored for about 24 hours in 3-ml volumes inside a lead container to allow for pyrogen testing prior to the administration to patients. This specific activity corresponded to an initial dose rate of approximately 4300 rads/hour to the IgG and a dose of about 100,000 rads for the 24-hour period.

Diluted IgG preparations with the same labeling ratio had specific activities of 2 mCi/ml and were stored in 15-ml volumes. This resulted in an initial dose rate of approximately 900 rads/hour and a dose of about 21,000 rads for the first 24 hours. Furthermore, for patients who were administered 30 mCi on Day 0 and 20 mCi on Day 5, diluted IgG preparations were stored for up to 6 days prior to the second infusion. The radiation dose to the IgG for this period of time was also approximately 100,000 rads. However, the effective half-life for both the first and second injections was nearly the same (fig 4) and longer than for either affinity-column purified or undiluted polyclonal antiferritin. Dilution with specific IgG, normal IgG, or 1% human serum albumin prolonged the effective half-life by the same factor and did not alter tumor uptake. Human serum albumin has, therefore, become the dilutant of choice. Administration of affinity-column purified IgG diluted with normal IgG following the labeling procedure to one patient did not show an increased effective half-life, suggesting again damage caused by the affinity-column purification procedure.

The relationship between tumor activity and volume implies that in future treatments the administered activity can be optimized to further reduce TBI and, at the same time, maintain maximum tumor irradiation.

Four patients with tumor volumes ranging from 2290 to 3020 cm^3 had reduced tumor uptake of radiolabeled antiferritin. Reduced tumor uptake was not related to administered activities or antiserum IgG preparations. The administered activities of ^{131}I-labeled antiferritin IgG for these patients ranged from 30 to 130 mCi and included polyclonal and affinity-column purified preparations. The data in table 2 and figure 6 indicate that the specific activities in the tumors of these patients were nearly the same. However, the number of patients with tumor volumes of ≥ 2290 cm^3 was too small to derive a tumor volume-versus-activity curve for them or to relate them meaningfully to the curve for the remaining 18 patients (fig 7).

Our noninvasive tumor localization measurements, based on gamma camera images, yield no information about binding sites and mechanisms of the administered radioimmunoglobulin in relation to ferritin present in the tumor. Elucidation of these important processes must be provided by experimental studies. Regardless of the mechanism of binding in vivo, we have shown reproducibly that there is localization of antiferritin in hepatoma and that for most patients the activity deposited in the tumor is linearly proportional to tumor volume.

The present treatment regimen of administering 30 mCi on Day 0 and 20 mCi on Day 5 is based on the result that 30 mCi of radiolabeled antiferritin saturates hepatic tumors to the same level as larger administered activities and that activities of 50 mCi were well-tolerated by most patients (3). The dosimetric studies of ^{131}I-labeled antiferritin IgG presented in this report have led to our present studies using four cycles of ^{131}I-labeled polyclonal antiferritin IgG. The results of these studies will be reported. It was evident from our present studies that dosimetric investigations of tumor-dose kinetics provided the foundation for preparative and administrative aspects of radiolabeled antibody treatment.

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Therapeutic Implications of a Stem Cell Model for Human Breast Cancer: A Hypothesis[1]

P. Selby,[2,*] J.-P. Bizzari,[3] and R. N. Buick[3,4]

A model for human breast cancer is proposed in which a subpopulation of relatively undifferentiated tumor stem cells gives rise to clones of cells by a process of exponential division. The progeny progressively become more differentiated but lose their proliferative potential. The model implies that breast cancers are heterogeneous with respect to differentiation features such as hormone receptors, both between different clones and within individual clones. Therefore, endocrine therapies are preferentially directed towards differentiated cells and are unlikely to be curative either in advanced disease or in the adjuvant setting. The reduction of differentiated cells by hormonal maneuvers may leave a population of more actively dividing tumor stem cells which are more sensitive to chemotherapy. Hormonal therapy followed by cytotoxic chemotherapy may be more likely to kill tumor stem cells than either form of therapy given alone. [Cancer Treat Rep 67:659–663, 1983]

The Stem Cell Concept

In normal renewal tissues, such as bone marrow or gut epithelium, it is believed that differentiated cells are continually replaced by a population of stem cells which have high proliferative potential and the capacity to renew themselves (1–3). A hierarchy is suggested to exist from the stem cell to the differentiated, nondividing cell. As cells move down that hierarchy, they express genes controlling cell differentiation, but progressively lose their proliferative potential (1,2). The stem cell concept may be applied to tumors (4–7). In this case, it is proposed that a subpopulation of tumor stem cells are responsible for tumor growth or regrowth after treatment, for metastases, and, in the laboratory, for tumor transplantation. Such tumor stem cells must have the capacity to produce a large number of progeny and to renew themselves.
of progeny and to renew themselves.

Several lines of evidence suggest that the stem cell concept may be applied to human tumors (8). Human tumor cells can differentiate in vivo and in vitro (9–11), and some evidence exists that proliferative activity and differentiation are inversely related (10,12). Fractionation of human tumor cells on the basis of size and density demonstrates that proliferative activity and potential are restricted to subpopulations (10,13). Clonal markers in hematologic malignancies show that such tumors originate from a multipotential stem cell, although most of the tumor cells consist of differentiated progeny (14,15).

The study of human tumor stem cells has been facilitated by the recent development of methods for growing colonies of human tumor cells in semisolid media (16–18). The proportion of cells within a tumor capable of colony formation is low, which may indicate that only a small proportion of cells within tumors have sufficient proliferative potential to form colonies, but may also reflect inadequate culture conditions in the assays. The relationship of colony-forming cells to stem cells is unclear, but some studies have detected cells with self-renewal capacity within colonies (8,19–21).

A stem cell hierarchy model for human epithelial tumors has recently been presented (22), and studies of the proliferation and differentiation of ovarian carcinoma ascites cells have supported its validity (10, 13,23).

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A Stem Cell Model for Human Breast Cancer

Breast cancer is a hormone-responsive tumor in which a variety of endocrine maneuvers may produce palliative effects in about one-third of patients with advanced disease. Breast cancer is heterogeneous with respect to the presence of cytoplasmic steroid hormone-receptor proteins, and those tumors containing receptors appear more likely to respond to endocrine therapies (24). We suggest a stem cell renewal and differentiation model for human breast cancer. A schematic cell differentiation hierarchy is presented in figure 1. The tumor stem cell has the ability to self-renew, and is therefore capable of regrowing or transplanting the tumor to a metastatic site. A proportion of cell divisions yield cells committed to a differentiation pathway resulting in an expanding clone of cells which are progressively more differentiated, but which also lose their proliferative potential. In the model, cells are heterogeneous for hormone-receptor content, and the receptor-positive cells are more differentiated and have less proliferative potential than receptor-negative cells.

There is evidence which would be compatible with such a model. Estrogen and progesterone receptors are associated with more differentiated tumors (25,26). In some studies, it has been found that the presence of receptors is inversely proportional to the number of cells actively proliferating within a tumor (12,27,28), and that more rapidly growing cell lines tend to be receptor-negative (29). When human breast cancer cells are fractionated on the basis of cell size, it appears that estrogen receptors are only found within a subpopulation of relatively large cells (30). In addition, immunofluorescent staining of human breast cancer cells for receptors suggests heterogeneity within a tumor (31).

It has been proposed that by the time human tumors have progressed to a clinically detectable size, they contain a number of clones (32–35). Clonal heterogeneity has been shown for a variety of experimental and human tumors (33–35). Therefore, it seems plausible that a human breast cancer may contain a variety of clones of differing receptor content and differing hormone responsiveness (fig 2). The existence of heterogeneity of receptor content *between* clones does not exclude heterogeneity of receptor content-*within* clones based upon a stem cell differentiation hierarchy. A model combining these two sources of heterogeneity is shown in figure 3.

The differentiation pathways of normal breast epithelium to ductal, alveolar, and milk protein-producing cells are complex and poorly understood (36,37). The changes that occur at puberty, during lactation, and during the menstrual cycle are influenced by hormones, although the specific receptors are not readily demonstrated in normal breast tissue (38). The model for a differentiation hierarchy in breast cancer is undoubtedly an oversimplification which may serve as a basis for discussion of therapeutic implications, and for further work.

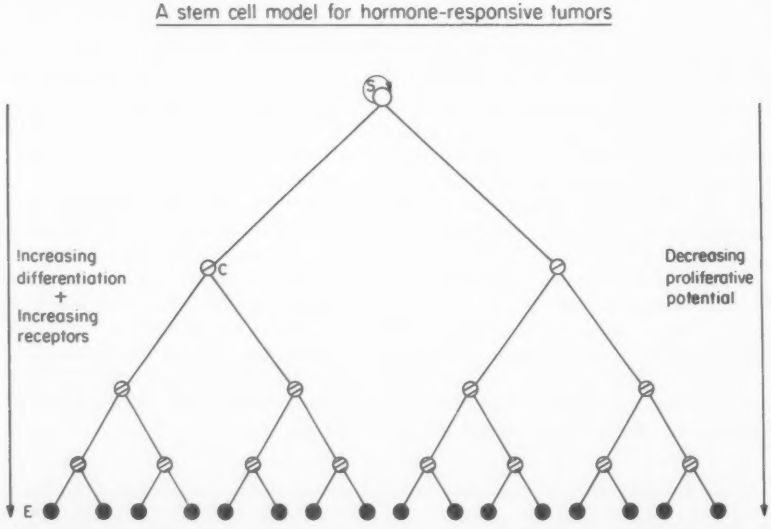

FIGURE 1.—The tumor stem cell, S, is capable of self-renewal and can give rise to a large number of progeny. It does not express differentiation markers such as hormone receptors. C is a more differentiated cell which retains some proliferative potential. E is an end cell which is differentiated but has lost the ability to divide.

FIGURE 2.—In this clonal model, the tumor consists of a heterogeneous collection of clones, some which may express differentiation markers such as hormone receptors (a), some which express no such markers (b), and some which are of intermediate differentiation (c).

Therapeutic Implications

Stem cells are the crucial target for any therapy which seeks to cure or substantially control cancer (5). Stem cells may differ from their progeny in a number of ways, notably proliferative status and degree of differentiation. These differences may be important in determining their relative sensitivity to treatment. In hormone-responsive tumors, a hormone maneuver may influence the receptor-positive, differentiated progeny preferentially and be relatively less effective against stem cells.

The failure of hormonal maneuvers to delete stem cells may underlie the eventual emergence of tumors that are unresponsive to hormones. The cellular mechanisms of hormone resistance which develop in unresponsive tumors are poorly understood. In some murine tumors, the development of hormone unresponsiveness may be associated with growth of autonomous cell populations of low hormone-receptor content (39). In other murine

FIGURE 3.—This model represents a synthesis of the clonal and stem cell models. Within each clone (A, B, and C), a stem cell (S) gives rise to a large No. of progeny, including differentiated, nondividing end cells (E). However, the degree of hormone-receptor expression differs between clones, eg, C > B > A for all levels of the cellular hierarchy.

tumors, karyo-typically distinct cell populations may emerge, but their receptor content may not be low (40). In human breast cancer, data are few, but clinical hormone resistance is not necessarily associated with reduced receptor content (41). The existence of substantial physicochemical heterogeneity among receptors (42), and of intact, but nonfunctional receptors (43), complicates the clarification of the mechanisms underlying acquired hormone resistance. Whatever these mechanisms may prove to be, preferential activity of endocrine treatments against nonstem cells could be important in allowing the emergence of the resistant cells. In human breast cancer, this would explain the failure of endocrine therapies to cure responsive, advanced disease. It would also predict that while adjuvant hormonal maneuvers might be expected to delay disease recurrence, they should not be expected to increase the proportion of cured patients. Studies of adjuvant endocrine therapy of breast cancer have so far failed to convincingly demonstrate any significant increase in overall patient survival as a result of treatment (44), with the possible exception of the premenopausal group of patients > 45 years of age in the ovarian ablation-prednisone study carried out in Toronto (45).

In stem cell systems, removal of differentiated progeny results in stimulation to division of immature precursor stem cells. This has been directly shown for human hemopoietic stem cells after bone marrow transplantation (46). It seems possible that such mechanisms could continue to operate in breast cancer so that successful endocrine treatment might result in suppression of the differentiated cells, and also in a more rapid division of the undifferentiated stem cells. This stem cell population might then be more sensitive to proliferation-dependent cytotoxic agents (47). The hypothesis suggests therapeutic benefit from sequential chemotherapy for those patients who have responded to endocrine therapy. It is of interest that some preliminary studies of sequential hormonal-cytotoxic chemotherapy have shown encouraging results (48). These results have been attributed to a direct stimulation of cell division by hormones rendering cells more chemosensitive. However, the results might be explained equally well by the stem cell feedback mechanism described above.

CONCLUSION

We have proposed a stem cell model for human breast cancer which can explain some of the clinical behavior of the disease and provide a basis for the investigation of new approaches to therapy. The model is testable by the analysis of the relationships between hormone receptors, other differentiation markers, and cellular proliferative activity and potential. If such studies in the laboratory provide support for the model, then clinical testing would be justified.

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Synthesis, Antitumor Activity, and Cardiac Toxicity of New 4-Demethoxyanthracyclines[1]

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The new anthracycline glycosides 4-demethoxy-4′-deoxydaunorubicin and 4-demethoxy-4′-*O*-methyldaunorubicin, synthesized by coupling 4-demethoxydaunomycinone with 1-chloro-derivatives of protected 4-*O*-methyl and 4-deoxydaunosamine derivatives, have been converted into the corresponding doxorubicin analogs. The new compounds have been compared for antitumor effect with the parent drugs and with the previously described 4-demethoxydaunorubicin, 4-demethoxy-4′-epidaunorubicin, and their doxorubicin analogs. All of the new analogs were more cytotoxic against HeLa cells in vitro and were more toxic and more potent in mice than the parent drugs. Comparison at optimal antitumor doses showed that the new analogs were as active as the parent drugs against ascitic P388 leukemia and disseminated Gross leukemia. They were also active when administered orally. The new doxorubicin analogs were slightly more active than doxorubicin against ascitic L1210 leukemia and were markedly more active against disseminated L1210 leukemia. In a parallel activity-cardiotoxicity test in C3H mice repeatedly treated iv, 4-demethoxydoxorubicin, 4-demethoxy-4′-epidoxorubicin, 4-demethoxy-4′-*O*-methyldoxorubicin, and 4-demethoxy-4′-deoxydoxorubicin showed antitumor activity against mammary carcinoma without inducing the typical myocardial lesions observed after doxorubicin treatment. 4-Demethoxy-4′-*O*-methyldoxorubicin, because of its high antitumor effectiveness, lack of cardiac toxicity in mice, and activity by the oral route, deserves further study. [Cancer Treat Rep 67:665–673, 1983]

During recent years a large number of analogs of daunorubicin (DNR) and doxorubicin (DX) have been prepared by chemical modification of the natural antibiotics, by total synthesis and glycosidation of modified anthracyclinones, and by synthesis of new glycosides containing the aminosugars related to daunosamine.

Structure-activity relationship studies have revealed that the C-4 position in the chromophore as well as the C-4′ position in the sugar moiety can be modified to give analogs endowed with favorable pharmacologic properties. Among the analogs modified in the aglycone moiety, 4-demethoxydaunorubicin (4-demethoxyDNR) showed higher potency than DNR in a spectrum of experimental mouse tumors (1), activity by oral administration (2), high activity against some experimental mouse leukemias (3), and lower cardiotoxicity than the parent compound in mice at equiactive doses and in rabbits at equimyelotoxic doses (4,5). On the basis of these results, 4-demethoxyDNR has been introduced into clinical trials (6,7). Similar biologic activity is shown by 4-demethoxydoxorubicin (4-demethoxyDX) (4,8,10). On the other hand, among the analogs modified in the sugar moiety, 4′-epidoxorubicin (4′-epiDX) was found to be less toxic than DX both in experimental models (11) and in patients (6); 4′-*O*-methyldoxorubicin (4′-*O*-methylDX) and 4′-deoxydoxorubicin (4′-deoxyDX) showed reduced cardiac toxicity (4) and higher activity than DX against human colon carcinoma transplanted in nude mice (12) and against experimental mouse colon tumors (13,14).

Since it was of interest to investigate the effect of modifications at the C-4′ position in the 4-demethoxyglycosides, we herein describe the synthesis, antitumor

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activity, and cardiac toxicity in mice of 4-demethoxy-4′-*O*-methyldaunorubicin (4-demethoxy-4′-*O*-methylDNR), 4-demethoxy-4′-deoxydaunorubicin (4-demethoxy-4′-deoxyDNR), 4-demethoxy-4′-*O*-methyldoxorubicin (4-demethoxy-4′-*O*-methylDX), and 4-demethoxy-4′-deoxydoxorubicin (4-demethoxy-4′-deoxyDX) (table 1). The synthesis of 4-demethoxy-4′-epidaunorubicin (4-demethoxy-4′-epiDNR) and of its doxorubicin analog 4-demethoxy-4′-epidoxorubicin (4-demethoxy-4′-epiDX) has already been reported (8).

The biologic activity of the new analogs was compared with that of DNR, DX, 4-demethoxyDNR, and 4-demethoxyDX. The results showed that the new 4-demethoxyglycosides are more toxic and more potent in vivo than the parent compounds, are active also after oral administration, and are highly effective against some experimental leukemias. In addition, all of the 4-demethoxyDX analogs showed antitumor activity against the mouse mammary carcinoma and markedly lower cardiotoxicity than DX.

MATERIALS AND METHODS

In vitro tests.—The colony-inhibition test was carried out, as previously described (9), on HeLa cells.

Animals.—Inbred DBA/2, C3H/He and first generation hybrid (C57BL/6 × DBA/2)F_1 (BDF_1) and (BALB/c × DBA/2)F_1 (CDF_1) adult mice of both sexes were obtained from Charles River Italia (Calco, Como, Italy). The animals were 2–3 months old, weighed 20–25 g, and were kept under standard laboratory conditions.

Tumors.—Ascitic P388 and L1210 leukemias were maintained by serial passage in DBA/2 mice, according to Geran et al (15). For experimental purposes, P388 leukemia cells (1×10^6/mouse) were inoculated ip and L1210 leukemia cells (1×10^5/mouse) were injected either ip or iv in BDF_1 or CDF_1 mice. Gross leukemia (16) was maintained by serial iv passage of a homogenate of spleen and peripheral lymph nodes of leukemic mice in C3H/He syngeneic mice. The experiments were carried out in the same strain (2×10^6 cells/mouse). In the case of mammary carcinoma, 20×10^6 cells from a tumor at third generation were inoculated sc, as previously described (17), in C3H/He female mice. Each experimental group consisted of at least eight animals.

Drug administration.—The compounds were dissolved in distilled water and were administered either ip, iv, or orally (by stomach intubation) in a volume of 10 ml/kg of body weight. The treatment schedules are reported in the Results section.

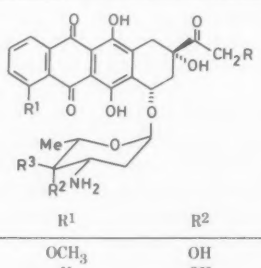
Evaluation of antitumor activity.—For P388, L1210, and Gross leukemias, the comparison of drug effectiveness was based on the increase in median survival time (MST) with the dose range used compared to untreated controls. The number of long-term survivors (LTS) refers to the surviving mice at the end of the experiment (60 days). For mammary carcinoma, tumor growth was assessed by caliper measurement, and tumor weight (mg) was calculated according to the formula:

$$w = d^2 \times D/2,$$

where d is the minimum and D is the maximum diameter (15).

Evaluation of toxicity.—Toxicity was established on the basis of the macroscopic necropsy findings evaluated in all dead mice. The main findings were spleen and liver size reduction and hemorrhagic degeneration of the intes-

TABLE 1.—Chemical structure of 4-demethoxyanthracyclines



Compound	R	R^1	R^2	R^3	Molecular weight*
DNR	H	OCH_3	OH	H	563.98
4-DemethoxyDNR	H	H	OH	H	533.97
4-Demethoxy-4′-epiDNR	H	H	H	OH	533.97
4-Demethoxy-4′-*O*-methylDNR	H	H	OCH_3	H	547.97
4-Demothoxy-4′-deoxyDNR	H	H	H	H	517.97
DX	OH	OCH_3	OH	H	579.98
4-DemethoxyDX	OH	H	OH	H	549.97
4-Demethoxy-4′-epiDX	OH	H	H	OH	549.97
4-Demethoxy-4′-*O*-methylDX	OH	H	OCH_3	H	563.97
4-Demethoxy-4′-deoxyDX	OH	H	H	H	533.97

* As hydrochlorides.

tinal mucosa, as previously reported (17). In mammary carcinoma experiments, non-tumor-bearing C3H female mice were treated in parallel with the tumor-bearing mice, and were observed for 3 months after the beginning of treatment for evaluation of lethality. One month after the last treatment, two to five mice in each group were killed, and hearts were removed and fixed in paraformaldehyde (4% in 0.1 M phosphate buffer at pH 7.3). Histologic examination of semithin sections was carried out as previously described (18). Myocardial lesions were graded according to severity and extension. The mean value is the severity degree × extension degree. The histologic examinations were blind.

RESULTS

Synthesis of the New Compounds[5]

4-Demethoxy-4′-O-methylDNR.—A solution of 3.68 g of 4-demethoxydaunomycinone (1) in 170 ml of anhydrous methylene chloride containing 1.4 g of 1-chloro-4-*O*-methyl-*N*-trifluoroacetyldaunosamine (19) was stirred in the presence of molecular sieve (15 g, 4 Å; Merck) and 1.48 g of silver trifluoromethanesulfonate. After 30 minutes at room temperature, the reaction mixture was neutralized with a saturated aqueous solution of sodium bicarbonate, and the organic phase, after being separated, was evaporated under vacuum to obtain a residue. The residue obtained was dissolved in 30 ml of acetone and treated with 200 ml of 0.1 N aqueous sodium hydroxide. After 5 hours at 0°C the solution was adjusted to pH 8.3 and extracted with methylene chloride. The residue, obtained by evaporation of the organic solvent, was purified on columns of silica gel buffered at pH 7 with phosphate buffer M/15, using the solvent system chloroform-methanol-water (10:2:0.2 vol/vol). The eluate, containing the pure 4-demethoxy-4′-*O*-methylDNR, was diluted with water and the organic phase was separated, washed with water, and evaporated to small volume; it was thereafter acidified to pH 5 with 0.1 N methanolic hydrogen chloride. The addition of excess diethyl ether yielded 1 g of product, as hydrochloride, melting point, 189°C–190°C (dec.); FD-MS: m/z 511 ($M^+\cdot$); 368 (aglycone). ^{1}H-NMR (DMSO-d_6): 1.21 (d, C*H*$_3$-C-5′); 2.27 (s, C*H*$_3$CO); 3.48 (s, C*H*$_3$O-C-4′); 4.88 (broad s, C*H*-7); 5.27 (broad s, C*H*-1′); 13.26 and 13.47 (two s, phenolic OH); thin-layer chromatography (TLC) on kieselgel plates (Merck F 254) solvent system chloroform-methanol-water (10:2:0.2 vol/vol): R_f 0.37. The high-performance liquid chromatographic (HPLC) flux rate was 1.5 minutes; retention time was 22 minutes.

Elemental analysis found (%):	C	56.12;	H	5.09;	N	2.32;	Cl	6.49
$C_{27}H_{30}O_9NCl.1.75\ H_2O$ calculated (%):		55.77;		5.82;		2.41;		6.11

4-Demethoxy-4′-O-methylDX.—The conversion of 4-demethoxy-4′-*O*-methylDNR to DX analog was performed following a procedure already described for the chemical transformation of DNR to DX (20). A solution of 0.5 g of 4-demethoxy-4′-*O*-methylDNR in a mixture of methanol (8 ml) and dioxane (20 ml) was treated with bromine to form the 14-bromoderivative. Treatment of the 14-bromoderivative with an aqueous solution of sodium formate at room temperature for 18 hours gave 4-demethoxy-4′-*O*-methyldoxorubicin, which was extracted from the reaction mixture by extraction with *n*-butanol. The organic solvent was evaporated to small volume and thereafter acidified to pH 5 with 0.1 N methanolic hydrogen chloride. The pure precipitated crystalline product (0.31 g) was collected by filtration; melting point, 164°C–165°C (dec.); FD-MS: m/z 527 ($M^+\cdot$); 384 (aglycone): ^{1}H-NMR (DMSO-d_6): 1.21 (d, C*H*$_3$-C-5′); 3.38 (s, C*H*$_3$-O-C-4′); 4.57 (s, C*H*$_2$-14), 4.91 (broad s, C*H*-7), 5.28 (broad s, C*H*-1′), 13.28 and 13.49 (two s, phenolic OH); TLC on kieselgel plates (Merck F 254) solvent system chloroform-methanol-water (10:2:0.2 vol/vol): R_f 0.18. The HPLC flux rate was 1.5 ml/minute; retention time was 10 minutes.

Elemental analysis found (%):	C	53.10;	H	5.42;	N	2.21;	Cl	6.70
$C_{27}H_{30}O_{10}NCl.1.75\ H_2O$ calculated (%):		54.27;		5.67;		2.34;		5.94

4-Demethoxy-4′-deoxyDNR.—The coupling reaction of 4-demethoxydaunomicinone (11.2 g) with 1-chloro-4-deoxy-*N*-trifluoroacetyldaunosamine (21) (7.6 g) is performed as described for the synthesis of 4-demethoxy-4′-*O*-methylDNR. The pure 4-demethoxy-4′-deoxyDNR (3.7 g), isolated as the hydrochloride, was obtained after chromatographic purification: melting point, 151°C–153°C (dec.); FD-MS: m/z 481 ($M^+\cdot$), 368 (aglycone); ^{1}H-NMR (DMSO-d_6): 1.18 (d, C*H*$_3$-C-5′), 2.26 (s, C*H*$_3$CO), 4.92 (broad s, C*H*-7), 5.36 (broad s, C*H*-1′), 13.28 and 13.53 (two s, phenolic OH); TLC on kieselgel plates (Merck F 254) solvent system chloroform-methanol-water (13:6:1 vol/vol): R_f 0.44. The HPLC flux rate was 2.3 ml/minute; retention time was 15′ 50.

Elemental analysis found (%):	C	57.17;	H	5.81;	N	2.50;	Cl	6.49
$C_{26}H_{28}O_8NCl.1.23\ H_2O$ calculated (%):		57.70;		5.69;		2.58;		6.55

[5]Melting points, obtained with a SMP-20 apparatus (Buchi), are uncorrected. Mass spectra were obtained with a Varian-Mat 311-A spectrometer equipped with a combined EI/FI/FD source. H-NMR spectra were recorded at 200 MHZ with a Varian XL-200 NMR spectrometer. HPLC analysis was performed using a Waters ALC-202 apparatus; experimental conditions: column μBondapak C_{18}: isocratic elution mobile phase water-acetonitrile (69:31) at pH 2 with 10% H_3PO_4. For determination of the elemental composition, the samples were dried at 80°C under vacuum (0.2 mm Hg) for 15 hours and subsequently equilibrated in the air (23°C and 55% relative humidity). After 30 minutes constant weight was reached, the weight increase corresponding to adsorbed water. Elemental analyses were performed on the equilibrated samples.

4-Demethoxy-4′-deoxyDX.—Operating as described for 4-demethoxy-4′-*O*-methylDX, 2.2 g of 4-demethoxy-4′-deoxyDNR gave 0.93 g of 4-demethoxy-4′-deoxyDX: melting point, 230°C (dec.); FD-MS: m/z 497 ($M^+ \cdot$), 384 (aglycone); ^{1}H-NMR (DMSO-d_6): 1.15 (d, C*H*$_3$-C-5′); 4.57 (s, C*H*$_2$-14); 4.91 (broad s, C*H*-7), 5.34 (broad s, C*H*-1′), 13.27 and 13.50 (two s, phenolic OH). TLC on kieselgel plate (Merck F 254) solvent system chloroform-methanol-water (13:6:1 vol/vol); R_f 0.3. The HPLC flux rate was 2.3 ml/minute; retention time: 6′ 70.

Elemental analysis found (%):	C	58.28;	H	5.38;	N	2.50;	Cl	6.64
$C_{26}H_{28}O_9$ NCl calculated (%):		58.48;		5.28;		2.62;		6.65

Biologic Activity

Data reported in table 2 show that all the new 4-demethoxyanthracyclines tested were more cytotoxic than DNR and DX for HeLa cells cultured in vitro. Against the P388 ascitic leukemia in mice, the new analogs, administered ip on Day 1, were more toxic and more potent than the parent compounds. At the maximal tolerated dose (MxTD) the compounds were about as active as DNR and DX (table 3).

The new DX analogs described here were tested in mice bearing L1210 leukemia in an ascitic form (inoculated ip) or in a disseminated form (inoculated iv). Drugs were administered on Day 1, ip or iv, respectively. The data obtained at the optimal dose, which is in general the MxTD, and at the minimal toxic dose (MinTD) are shown in table 4.

The new analogs, at the MxTD, were slightly more active than DX against the ascitic L1210 leukemia. Against the disseminated L1210 leukemia, both 4-demethoxyDX and 4-demethoxy-4′-*O*-methylDX were markedly more effective than DX. In fact, at the MxTD of 16 mg/kg, DX produced an increase in lifespan not superior to 66%, and did not cure any of the treated mice. 4-DemethoxyDX and 4-demethoxy-4′-*O*-methylDX, at the nontoxic doses of 2.7 and 1.7 mg/kg, respectively, markedly increased the lifespan of the tumor-bearing mice and cured about 50% of them, as can be observed by the number of survivors at 60 days after the beginning of the experiment.

TABLE 2.—Colony-forming ability of cultured HeLa cells after treatment with 4-demethoxy derivatives of DNR and DX*

Compound	Concentration (ng/ml) required for 50% inhibition (± SE)
DNR	11.6 ± 0.76
4-DemethoxyDNR	5.2 ± 1.01
4-Demethoxy-4′-epiDNR	5.5
4-Demethoxy-4′-*O*-methylDNR	2.5
4-Demethoxy-4′-deoxyDNR	2
DX	10 ± 0.40
4-DemethoxyDX	1
4-Demethoxy-4′-epiDX	3
4-Demethoxy-4′-*O*-methylDX	1
4-Demethoxy-4′-deoxyDX	0.4

* HeLa cells were exposed to the drugs for 24 hrs.

The results obtained against the disseminated Gross leukemia are shown in table 5. Compounds were administered iv on Day 1. At their individual optimal doses, all of the new analogs were about as active as the parent compounds. The new analogs were compared with 4-demethoxyDNR in mice bearing Gross leukemia and treated orally on Day 1 or on Days 1, 2, and 3 after tumor inoculation. Results are reported in table 6. All of the compounds tested were also active when administered orally. By comparing the data reported in table 5 with those reported in table 6, it can be observed that when treatment was given orally on Day 1, the optimal doses were equal to or twice the optimal iv doses. To know the relationship between antitumor activity and cardiac toxicity, the new DX analogs were compared with DX against the mammary carcinoma of C3H mice. Treatment was given iv once a week for 4 weeks, starting when tumors were palpable. Non-tumor-bearing C3H mice were treated in parallel and observed for 90 days for the evaluation of general toxicity; two to five mice per group were killed 1 month after the last treatment, and heart lesions were evaluated histologically. Results are reported in tables 7 and 8.

DX was toxic at the dose of 9 mg/kg. At lower doses (6 or 7.5 mg/kg/day) it inhibited tumor growth by 64%–93%.

Cardiac lesions were evident in all of the DX-treated mice, and the severity of the lesions increased with increasing dose, both in the atrium and in the ventricles. In contrast, 4-demethoxyDX and the other new DX analogs investigated produced only minimal scattered cardiac lesions, the severity of which did not increase with increasing dose. With regard to the antitumor activity, all of the compounds were active but only 4-demethoxy-4′-epiDX and 4-demethoxy-4′-*O*-methylDX, at tolerated doses, inhibited tumor growth by > 50% and increased the survival time of the tumor-bearing mice.

DISCUSSION

The availability of optically active 4-demethoxydaunomycinone following an efficient synthetic procedure developed in our laboratories has allowed the extension of the 4-demethoxy series to compounds containing different aminosugars.

The silver triflate glycosidation procedure appeared to be successful and stereoselective as previously reported, giving yields of α-glycosides in the range of 40%–60% on the basis of the glycosyl halide reactant.

The results obtained in vitro with the new compounds investigated confirm the high cytotoxic properties of the 4-demethoxyanthracyclines, when compared with the

TABLE 3.—Effect of 4-demethoxyanthracyclines against ascites P388 leukemia *

Compound	Dose (mg/kg)†	T/C %‡	LTS§	No. of toxic deaths/ No. of mice treated¶
DNR	1.25	128	0/10	0/10
	2–2.9	168 (150–200)	2/68	0/68
	4–5	164 (131–205)	0/78	18/78
	6.6	138 (109–163)	0/48	11/48
4-DemethoxyDNR	0.25	114	0/10	0/10
	0.5	147 (180,114)	0/20	0/20
	0.75	188 (200,175)	0/20	4/20
	1.0	155 (130,180)	0/20	17/20
4-Demethoxy-4′-epiDNR	0.5	140	0/8	0/8
	0.8	150	0/8	0/8
	1.2	98 (95,100)	0/18	16/18
4-Demethoxy-4′-*O*-methylDNR	0.5	127	0/10	0/10
	0.8	150 (127,172)	0/20	0/20
	1	145	0/10	0/10
	1.3	95	0/10	10/10
4-Demethoxy-4′-deoxyDNR	0.5	154	0/10	0/10
	1	186	0/10	0/10
	2	210	1/10	2/9
	4	63	0/10	10/10
DX	2.5	162 (150,173)	0/20	1/20
	4–5	191 (162–218)	0/60	0/60
	6.6	203 (190–220)	2/26	0/26
	10	244 (200–315)	7/56	2/56
4-DemethoxyDX	0.25	168	0/10	0/10
	0.5	198 (195,200)	1/20	0/20
	0.75	195	0/10	0/10
	1	209	2/10	2/10
	2	100	0/9	9/9
4-Demethoxy-4′-epiDX	0.25	154 (164,143)	0/20	0/20
	0.5	168 (162,173)	1/20	0/20
	0.75	195	2/10	0/10
	1	162	0/10	8/10
4-Demethoxy-4′-*O*-methylDX	0.4	180	0/10	0/10
	0.6	215	0/10	0/10
	1	250	0/7	1/7
	1.5	245	2/10	4/10
4-Demethoxy-4′-deoxyDX	0.04	130	0/8	0/8
	0.12	170	0/8	0/8
	0.25	200 (195,205)	0/16	0/16
	0.5	235	0/8	0/8
	1	83 (75,90)	0/16	13/16

* BDF_1 or CDF_1 mice received 10^6 ascites cells ip on Day 0. MST of control mice was 10 days (range, 8–11) in 14 experiments.
† Drugs were injected ip as the hydrochloride on Day 1.
‡ MST of treated mice/MST of control mice × 100. Nos. in parentheses = data obtained in single experiments (,) or range (–).
§ No. of LTS (> 60 days)/No. of mice treated.
¶ Evaluated as described in the Materials and Methods section.

parent antibiotics. A more marked increase was found in the DX series than in the DNR series. In fact, the DNR analogs described were from two to six times more cytotoxic against HeLa cells than DNR, while the DX analogs were from three to 25 times more cytotoxic than DX.

A relationship was found between in vitro cytotoxicity and toxicity in mice, evaluated by necropsy findings on the tumor-bearing animals, as previously observed by us with a number of anthracyclines (22). On the contrary, the studies carried out in C3H mice treated iv repeatedly show that all of the 4-demethoxyDX tested, compared with DX at doses producing similar lethality in normal mice and similar inhibition of mammary tumor growth, was markedly less cardiotoxic than the parent compounds. These data confirm that substitution of the 4-methoxyl group in the anthracycline molecule with a hydrogen atom causes an increase in cytotoxicity, potency, and general toxicity, but it does not cause a paral-

TABLE 4.—Effect of 4-demethoxyanthracyclines against L1210 leukemia

Compound	Tumor* inoculum route	Treatment† Route	Treatment† mg/kg	T/C % ‡	LTS ‡	No. of toxic deaths/No. of mice treated ‡
DX	Ip	Ip	5	164 (166,161)	2/21	1/21
			10	174 (155-205)	6/31	9/31
	Iv	Iv	13	134 (120-150)	0/19	0/19
			16	154 (133-166)	0/30	2/30
4-DemethoxyDX	Ip	Ip	0.5-0.75	174 (166-189)	3/31	1/31
			1	133	0/11	11/11
	Iv	Iv	2.7	> 900, >750	11/20	0/20
			3.6	> 750	8/9	1/9
4-Demethoxy-4'-epiDX	Ip	Ip	0.75	189	2/10	0/10
			1	133	0/11	11/11
4-Demethoxy-4'-*O*-methylDX	Ip	Ip	1	187	1/10	1/10
			1.2	393	4/10	3/10
	Iv	Iv	1.7§	> 580	5/10	0/10
4-Demethoxy-4'-deoxyDX	Iv	Iv	1.2	191	0/10	0/10
			1.5	116	0/10	6/10

* BDF_1 or CDF_1 mice received 10^5 leukemia cells ip or iv on Day 0. MST of control mice inoculated ip was 8.8 days (range, 8-9) in 5 experiments. MST of control mice inoculated iv was 7.1 days (range, 6-8).

†Drugs were injected as the hydrocloride ip or iv on Day 1.

‡*See* table 3 for definitions.

§Maximal dose tested.

lel increase in cardiotoxic properties. The mechanisms which are at the basis of the cardiotoxic effect are not yet known and several possible biochemical mechanisms have been suggested, including free radical formation (23) and alterations in Ca ion membrane exchange (24,25). In addition, different pharmacokinetic properties can influence the degree of cardiotoxicity of anthracyclines (26). The 4-demethoxy derivatives of DX possessing different aminosugar residues described here are of extreme interest in this respect and will be further investigated for pharmacokinetic and biochemical properties. With regard to the antitumor effectiveness, in general the new compounds at their individual optimal dose retained the antitumor effectiveness of the parent compound against P388 and Gross leukemias. Outstanding results were obtained against the L1210 leukemia injected iv. In this experimental system, 4-demethoxyDX and 4-demethoxy-4'-*O*-methylDX were markedly more

TABLE 5.—Effect of 4-demethoxyanthracyclines against Gross leukemia *

Compound	Dose (mg/kg)†	T/C % ‡	LTS ‡	No. of toxic deaths/ No. of mice treated ‡
DNR	15	187 (175-216)	0/38	5/38
	22.5	210 (191-225)	1/38	9/37
4-DemethoxyDNR	3.3	233	0/8	0/8
4-Demethoxy-4'-epiDNR	2.2	200	0/10	0/10
	3.3	116	0/10	9/10
4-Demethoxy-4'-*O*-methylDNR	1.25	196 (183,208)	2/20	0/20
	1.5	100	0/10	9/10
4-Demethoxy-4'-deoxyDNR	1.5	166	0/8	0/8
	1.9	200	0/8	7/8
DX	13	204 (200-216)	2/36	2/36
	16.9	241 (216-266)	1/36	3/36
4-Demethoxy-4'-*O*-methylDX	1	208 (183,233)	0/18	0/18
	1.3	200 (192,208)	0/18	4/18
4-Demethoxy-4'-deoxyDX	1.2	179 (150,208)	0/20	0/20
	1.8	133	0/10	9/10

* C3H mice received 2×10^6 leukemia cells iv on Day 0. MST of controlled mice was 6 days in 9 experiments.

†Drugs were injected as the hydrochloride iv on Day 1.

‡*See* table 3 for definitions.

TABLE 6.—Effect of 4-demethoxyanthracyclines administered orally to mice bearing Gross leukemia*

Schedule of treatment (Day) †	Compound	Dose (mg/kg/day)	T/C% ‡	No. of toxic deaths/ total No. of mice treated ‡
1	4-DemethoxyDNR	3.3	150	0/8
		4.0	108	6/8
	4-Demethoxy-4′-epiDNR	5.0	150	0/10
		7.5	166	5/10
	4-Demethoxy-4′-*O*-methylDNR	1.3	150 (166,133)	1/20
		1.6	166 (166,166)	3/20
	4-Demethoxy-4′-*O*-methylDX	1.7–2.5	166	0/8
	4-Demethoxy-4′-deoxyDX	2.7	150 (142–158)	2/26
		5.4	133	4/6
1,2,3	4-DemethoxyDNR	1.9	162 (133,190)	1/20
		2.5	210	2/10
	4-Demethoxy-4′-*O*-methylDNR	0.66	183	0/10
		1	200	3/10

* C3H mice received 2×10^6 leukemia cells iv on Day 0. MST of control mice was 5.9 days (range, 5–6) in 10 experiments.
†Drugs were administered as the hydrochloride at the indicated days by stomach intubation.
‡ See table 3 for definitions.

effective than DX, which is in agreement with the previously reported effect of 4-demethoxyDNR (3). All of the DX derivatives were active against the mammary carcinoma. The highest activity at tolerated doses was observed after treatment with 4-demethoxy-4′-*O*-methylDX: 90% inhibition of tumor growth and 53% increase in lifespan at the dose of 0.75 mg/kg/day; the activity was equal to that of DX at the doses of 6 and 7.5 mg/kg/day.

Because of the high potency, lack of cardiac toxicity, and high antitumor effectiveness against leukemias and a solid tumor, this compound seems particularly promising and deserves further study. Finally, all of the compounds were active against Gross leukemia also after oral administration.

In conclusion, the main biologic properties identified in the past for 4-demethoxyDNR and 4-demethoxyDX are confirmed also for these derivatives, that is, increased cytotoxicity and potency, increased antitumor effectiveness against some experimental leukemias, and reduced cardiotoxicity at equiactive dose with respect to the parent compounds. This allows us to identify a "class" of anthracyclines, the 4-demethoxyanthracyclines, which have several properties in common. The present study shows that additional modifications in the chemical structure of 4-demethoxyanthracyclines can bring about additional favorable effects, such as the high effectiveness against mammary carcinoma investigated by us as a model of solid tumors.

TABLE 7.—Effect of new 4-demethoxy analogs of DX against advanced mammary carcinoma of C3H mice*

	New analog			DX		
Compound	Dose (mg/kg/day)†	Tumor weight (T/C%)‡	MST (T/C%)§	Dose (mg/kg/day)†	Tumor weight (T/C%)‡	MST (T/C%)§
4-DemethoxyDX	1	43	114	6	36	120
	1.5	ND¶	61	9	8	118
4-Demethoxy-4′-epiDX	0.6	39	126	6	31	123
	0.9	22	137	9	8	141
4-Demethoxy-4′-*O*-methylDX	0.4	36	123	6	9	145
	0.6	19	143	7.5	7	140
	0.75	10	153	—	—	—
4-Demethoxy-4′-deoxyDX	0.4	41	115	6	29	113
	0.6	16	94	9	5	144

* C3H female mice were inoculated sc with 20×10^2 cells from a 3rd-generation transplant of mammary carcinoma of C3H mice. The data obtained with the new analogs are reported in comparison with the data obtained after DX treatment in the same experiment.
†Drugs were administered as the hydrochloride once a wk for 4 wks iv, starting when tumors were palpable.
‡Average tumor weight of treated mice/average tumor weight of controls, × 100 (measured 1 wk after the last treatment).
§MST of treated mice/MST of controls, × 100.
¶Not determined due to toxicity.

TABLE 8.—Toxic deaths and cardiac toxicity of DX and 4-demethoxyDX analogs in non-tumor-bearing C3H female mice

Compound*	Dose (mg/kg/day)	No. of toxic deaths/ total No. of mice examined†	Atrial lesions		Ventricular lesions	
			No.‡	Grade§	No.‡	Grade§
—	—	0/13	0/14	0	4/15	0.1
DX	6	2/13	8/9	0.65	12/15	1.5
	7.5	2/3	4/4	1.3	5/5	1.5
	9	9/13	10/10	2.9	13/13	3.6
4-DemethoxyDX	1	1/5	1/5	0.1	5/5	0.5
	1.5	6/6	1/4	0.1	4/4	0.5
4-Demethoxy-4′-epiDX	0.6	0/3	0/5	0	0/5	0
	0.9	0/3	1/5	0.1	4/5	0.4
	1.3	6/8	0/2	0	2/2	0.7
4-Demethoxy-4′-*O*-methylDX	0.4	ND	ND	ND	ND	ND
	0.6	ND	ND	ND	ND	ND
	0.75	1/4	0/5	0	2/5	0.2
4-Demethoxy-4′-deoxyDX	0.4	1/5	0/5	0	1/5	0.1
	0.6	3/5	0/4	0	1/4	0.1

*Non-tumor-bearing C3H female mice were treated once a wk for 4 wks iv, in parallel with tumor-bearing mice reported in table 7. ND = not done.
†Mice were observed for at least 90 days after the start of treatment.
‡No. of mice showing lesions/total No. of mice examined.
§Average grade of lesions, evaluated according to Bertazzoli et al (18).

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Value of Prophylactic Cranial Irradiation Given at Complete Remission in Small Cell Lung Carcinoma[1,2,3]

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The optimal use of "prophylactic" cranial irradiation (PCI) in patients with small cell lung cancer remains undetermined. This study reviews the impact of PCI, given at complete remission (CR), on neurologic relapse in 172 consecutive patients with small cell lung cancer treated in three sequential chemotherapy protocols at the University of Maryland Cancer Center. In the first study of 38 patients, none received PCI. In the second study of 109 patients, the first 28 achieving CR were randomized to 3000 rad of PCI in ten fractions (PCI+) or to observation (PCI–). Thereafter, based on interim analysis, all patients achieving CR received PCI. In the third study, to date, 25 patients achieving CR have received PCI. Overall, 169 patients were evaluable for neurologic relapse, and 30 of 90 patients achieving CR received PCI. Among all patients with CR, with adjustment for disease extent, there was a significant delay to any neurologic relapse ($P = 0.01$) and cerebral metastases ($P = 0.02$) for PCI+ compared to PCI– patients. Among PCI– patients with CR, cerebral metastases alone occurred in 28% as the sole site and in 33% as the initial site, whereas cerebral relapse occurred prior to systemic relapse in only one PCI+ patient with CR. Patient survival, however, was not significantly altered by PCI. PCI at CR confers effective and worthwhile local control in the CNS, especially during periods of systemic response, and a small percentage of patients may benefit. Systemic drug resistance still determines overall survival. [Cancer Treat Rep 67:675–682, 1983]

Neurologic relapse remains a major cause of morbidity and mortality in small cell carcinoma of the lung (SCLC) despite the use of "prophylactic" cranial irradiation (PCI) used to treat intracranial micrometastases and the use of chemotherapeutic agents which cross the blood-brain barrier. To date, cytotoxic agents are not known to reduce the incidence of cerebral relapses, whatever their blood-brain barrier characteristics (1,2). In addition, the now routine use of combination chemotherapeutic regimens makes it difficult to assess the protective effect of individual agents. Although PCI substantially reduces the incidence of cerebral relapse, it has not improved overall survival (1,3–9).

Two important objectives for the application of PCI should be (*a*) the identification of patients who might potentially benefit from PCI and (*b*) the further characterization of the effects of PCI on the clinical course of SCLC, including the optimum timing for PCI and its effect on time to CNS relapse. One of the most likely groups of patients with SCLC to benefit from PCI could be those achieving complete remission (CR). First, they can be considered to have the lowest continuous risk for the formation of new metastases. Second, it would seem reasonable that the optimal time to give PCI is at CR, because in the presence of residual disease there is a potential risk of new cerebral metastases, whereas after CR has been attained this risk should fall providing that systemic CR is maintained. Third, patients achieving CR have sufficient prolongation of disease-free survival to derive benefit from any delay in the onset of CNS metastases; this could also possibly permit the recognition of survival benefits which might be conferred by PCI.

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Another objective of PCI given at the time of CR would be to determine that CNS relapses would not occur in the interval from initiation of chemotherapy until the achievement of CR.

The purpose of this paper is to address the foregoing objectives by an evaluation of 172 consecutive SCLC patients treated on three sequential University of Maryland Cancer Center (UMCC) protocols, in which PCI, although not initially routine, was later given to some, and then to all, patients when they had achieved CR.

METHODS

From June 1975 to June 1981, 172 consecutive patients with histologically proven SCLC were placed on three consecutive UMCC protocols (fig 1). These studies have been the subject of prior reports (9–13). Only patients with concurrent malignancies or those who had previously been treated with chemotherapeutic agents were not eligible for these protocols. In the first study (UMCC 7510), PCI was not used, since it was then unclear whether etoposide might cross the blood-brain barrier and reduce cranial metastases. In the subsequent study (UMCC 7705), patients achieving CR were initially randomized to receive PCI (PCI+) or to observation only (PCI–), to assess the effect of the timing of CNS irradiation on CNS metastases. However, an interim analysis revealed that none of 17 patients who received PCI had developed cerebral metastases, whereas four of the 15 who were observed and five of the 14 who refused randomization developed such metastases, and the randomization was discontinued. All patients who attained CR thereafter were given PCI. PCI is also given in the current successive study (UMCC 8020). Patients entered on UMCC 8020 after June 1981 were excluded from this analysis to allow a minimum of 10 months' follow-up from the start of chemotherapy. Patients who presented with intracranial metastases were considered separately. These patients received 3000 rad in ten fractions with therapy. In all studies where PCI was given, it was started within 14 days of the documentation of CR.

Both prior to treatment and for the documentation of CR, patients were intensively staged with clinical examination; bronchoscopy with brushings, washings, and biopsies; whole-lung tomography, computerized axial tomographic (CAT) scan, or both; CAT brain scan, with and without contrast; isotopic bone and liver scans; bilateral iliac crest bone marrow aspirations and biopsies; and complete hematologic and biochemical studies. The pretreatment and treatment characteristics of patients are shown in tables 1 and 2. The difference in the proportion of patients with limited disease in the PCI+ group is in part a reflection of the higher CR frequency seen among patients with limited disease. Therefore, the characteristics for the PCI– patients achieving CR are also shown in tables 1 and 2.

Standard criteria for response were used. CR was defined as the return to normal of all pretreatment abnor-

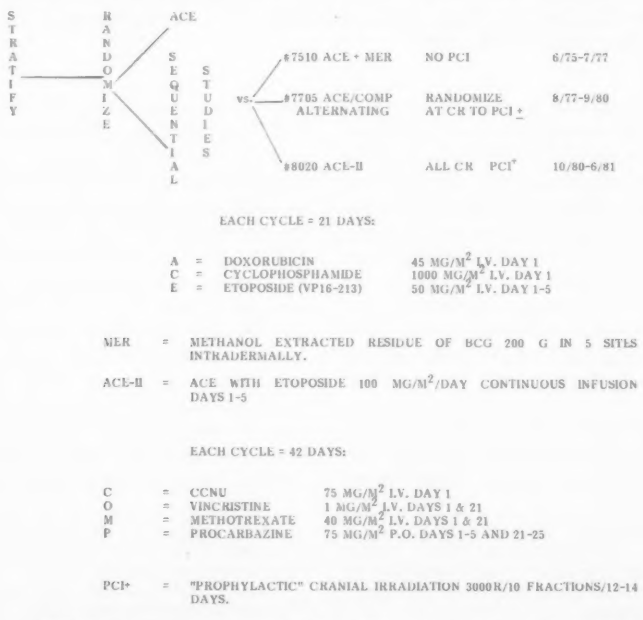
STRATIFY
RANDOMIZE
ACE
SEQUENTIAL
STUDIES
vs:
#7510 ACE + MER
NO PCI
6/75-7/77
#7705 ACE/COMP ALTERNATING
RANDOMIZE AT CR TO PCI +
8/77-9/80
#8020 ACE-II
ALL CR PCI+
10/80-6/81
EACH CYCLE = 21 DAYS:
A = DOXORUBICIN 45 MG/M2 I.V. DAY 1
C = CYCLOPHOSPHAMIDE 1000 MG/M2 I.V. DAY 1
E = ETOPOSIDE (VP16-213) 50 MG/M2 I.V. DAY 1-5
MER = METHANOL EXTRACTED RESIDUE OF BCG 200 G IN 5 SITES INTRADERMALLY.
ACE-II = ACE WITH ETOPOSIDE 100 MG/M2/DAY CONTINUOUS INFUSION DAYS 1-5
EACH CYCLE = 42 DAYS:
C = CCNU 75 MG/M2 I.V. DAY 1
O = VINCRISTINE 1 MG/M2 I.V. DAYS 1 & 21
M = METHOTREXATE 40 MG/M2 I.V. DAYS 1 & 21
P = PROCARBAZINE 75 MG/M2 P.O. DAYS 1-5 AND 21-25
PCI+ = "PROPHYLACTIC" CRANIAL IRRADIATION 3000R/10 FRACTIONS/12-14 DAYS.

FIGURE 1.—Sequential randomized studies of ACE combination chemotherapy vs ACE + additional treatment for SCLC.

TABLE 1.—SCLC: pretreatment characteristics*

	Overall	PCI+	All PCI-	CR only PCI-†
Total	172	30 (17)	142 (83)	60
Age in yrs				
Median	59	60	59	59
Range	29-79	48-71	29-79	48-76
Male:female	2.5:1	2:1	3:1	2:1
White:nonwhite	4:1	2:1	5:1	4:1
Performance status‡				
0-2	127 (74)	26 (87)	100 (70)	44 (73)
3,4	45 (26)	4 (13)	42 (30)	16 (27)
Extent of disease				
Limited	66 (38)	24 (80)	42 (30)	27 (45)
Extensive	106 (62)	6 (20)	100 (70)	33 (55)

* Unless specified otherwise, values = No. of patients (%).
†All patients receiving PCI (PCI+) achieved CR; therefore, a separate column for PCI- patients with CR.
‡ECOG criteria.

malities for a minimum of 30 days and no evidence of disease on any of the restaging evaluations. PR was defined as a minimum decrease of 50% in the cross perpendicular dimensions of each lesion, with a subjective improvement and no evidence of progression, lasting a minimum of 30 days. Progressive disease was defined as an increase of at least 25% in the size of lesions or the appearance of new lesions. Stable disease was defined as the absence of progressive disease, with no new lesions for a period of ≥ 60 days.

Limited disease was defined as disease confined to one hemithorax with or without bilateral hilar or mediastinal lymph node involvement and with or without ipsilateral supraclavicular lymph node involvement (single irradiation port). Extensive disease was defined as any involvement beyond the confines of the definitions of limited disease.

Meningeal carcinomatosis was defined as the demonstration of SCLC cells in cerebrospinal fluid during life or by histopathology at necropsy. Simultaneous occurrence

TABLE 2.—SCLC: treatment characteristics*

	Overall	PCI+	PCI-	PCI-CR
Total	172	30	142	60
Chemotherapy (*see* fig 1)				
UMCC 7510				
ACE	13 (8)	0 (0)	13 (9)	10
ACE + MER	25 (15)	0 (0)	25 (17)	14
UMCC 7705				
ACE; then COMP	54 (31)	9 (30)	45 (32)	17
ACE/COMP alternating	55 (32)	10 (33)	45 (32)	18
UMCC 8020				
ACE	11 (6)	5 (17)	6 (4)	1
ACE (high-dose etoposide)	14 (8)	6 (20)	8 (6)	0
Response to chemotherapy	169†	30	139†	60
No measurable disease (NMD)	1 (1)	1 (3)	0	0
CR	89 (53)	29 (97)	60 (43)	60
Partial remission (PR)	50 (29)	0	50 (36)	—
Stable disease	26 (15)	0	26 (19)	—
Progressive disease	3 (2)	0	3 (2)	—
Survival in mos from start of chemotherapy: median (range)				
CR/NMD	15 (2-72+)	15 (6-46+)	13 (2-72+)	13 (2-72+)
PR	10 (2-26)	—	10 (2-26)	—
Stable disease	5 (1-11)	—	5 (1-11)	—
Progressive disease	5, 6, 12	—	5, 6, 12	

* Unless specified otherwise, values = No. of patients (%).
†3 patients not evaluable for response, all receiving UMCC 8020: 1 lost to follow-up; 2 early deaths—1 on Day 6 (of cardiopulmonary arrest) and 1 on Day 34 (of sepsis).

of cerebral metastases with meningeal carcinomatosis was defined as the recognition of metastases or meningeal carcinomatosis within 30 days of one another. Two patients had relatively localized tumor in the extradural space and adjacent dura together with neurologic deficits; although this may have represented primary meningeal carcinomatosis, they were grouped separately.

A sole site of relapse was defined as the only identifiable site of SCLC relapse. An initial site of relapse was that site of relapse which preceded the appearance of other areas of relapse by at least 30 days. A primary or early CNS relapse was defined as one in which neurologic relapse was the sole site or the initial site of relapse. A secondary or later neurologic relapse was defined as one which occurred > 1 month from the time of relapse at other sites. Relapse data obtained from necropsy were included throughout this analysis. Inclusion or omission of these data would not alter the statistical analysis or conclusions, but their inclusion makes the data more complete.

Statistical Methods

The incidence of neurologic relapses in study subgroups was compared by the Fisher exact test method (14). The influence of PCI on the relative timing of neurologic relapses was assessed by the Mantel-Haenszel method of combining 2 × 2 tables (15). This method adjusted for the different weighting of limited- and extensive-disease patients in the PCI+ and PCI- groups. Time events such as time to first neurologic relapse were plotted by the life-table method, and times to neurologic relapse as well as survival were assessed by log-rank analysis (15).

RESULTS

Of the 172 patients analyzed only three were not evaluable for neurologic relapse because of loss to follow-up. No patients who achieved CR were found to develop neurologic relapse in the interval from start of therapy to documentation of CR. The sites and incidence of neurologic relapses and the relative timing of these relapses are shown in table 3 for all patients and in table 4 for patients who achieved CR. The median time to PCI was 3 months (range 1-9) and the median survival time following PCI was 13 months (range, 4-42+). The median time to neurologic relapse following PCI was 7 months (range, 2-14). Neurologic relapses occurred in 37% of patients overall (table 3), in 20% of PCI+ patients, and in 41% of PCI- patients (all response categories) (PCI+ vs PCI-, $P = 0.023$). In patients with CR (table 4) the respective incidences were 34% (overall), 20% (PCI+), and 42% (PCI-) ($P = 0.034$), and for cerebral metastases alone they were 24% (overall), 13% (PCI+), and 30% (PCI-) ($P = 0.067$). More important is the timing of CNS relapse and the analysis of time to relapse.

Among PCI- patients who experienced neurologic relapse, the relative timing of those relapses was 30% as sole site, 19% as initial site, and 49% together with or after other sites of relapse (table 3). In CR PCI- patients (table 4), the respective incidences of neurologic relapses were 36% (sole site), 23% (initial site), and 41% (later sites), and the incidences of cerebral metastases alone were 28% (sole site), 33% (initial site), and 39% (later sites). In contrast, no PCI+ patients had a neurologic or cerebral relapse as the only site of relapse, and cerebral relapse was the initial site in only one patient (tables 3 and 4), with the remainder of these relapses occurring together with or subsequent to systemic relapse. This delay in the occurrence of relapses after PCI is significant at the 0.01 level for all neurologic relapses and at the 0.02 level for cerebral metastases alone ("earlier" vs "later" or not at all, adjusted by the Mantel-Haenszel method for disease extent).

TABLE 3.—SCLC: neurologic relapses—all evaluable patients

	No. of patients (%)		
	Overall	PCI+	PCI-
No. evaluable	169	30	139
With neurologic relapse			*
All types	63/169 (37)	6/30 (20)	57/139 (41)
Specific types			
Cerebral metastases (M)	38 (60)	4 (67)	34 (60)
Meningeal carcinomatosis (MC)	17 (27)	2 (33)	15 (26)
M + MC simultaneously	6 (10)	0 (0)	6 (11)
Extradural/adjacent dura	2 (3)	0 (0)	2 (3)
Timing of neurologic relapse			
Total relapses	63	6	57
"Earlier"			
Sole site	19 (30)	0 (0)	19 (33)
Initial site	12 (19)	1 (17)	11 (19)
"Later"†	31 (49)	5 (83)	26 (46)
Indeterminant	1 (2)	0 (0)	1 (2)

*P = 0.023.

†Occurring together with or after other sites of relapse.

TABLE 4.—SCLC: neurologic relapses—CR patients only

	No. of patients (%)		
	Overall	PCI+	PCI−
No. evaluable	90	30	60
With neurologic relapse			
All types	31/90 (34)	6/30 (20)	25/60 (42)
Specific types			
Cerebral metastases (M)	22 (24)	4 (13)	18‡ (30)
Meningeal carcinomatosis (MC)	8 (26)	2 (33)	6 (24)
M + MC simultaneously	1 (3)	0	1 (4)
Timing of neurologic relapse relative to other sites of relapse			
All neurologic relapses	31	6	25
"Earlier"			
Sole site	9 (29)	0	9 (36)
Initial site	7 (23)	1 (17)	6 (24)
"Later"§	15 (48)	5 (83)	10 (40)
Cerebral metastases only	22	4	18
"Earlier"			
Sole site	5 (23)	0 (0)	5 (28)
Initial site	7 (32)	1 (25)	6 (33)
"Later"§	9 (41)	3 (75)	7 (39)

*$P = 0.034$.
†$P = 0.067$.
‡Includes 1 patient who developed MC 4 mos after M.
§Occurring together with or after other sites.

PCI+ and PCI- groups were also compared for the times from CR to the occurrence of any neurologic relapse (fig 2) or cerebral metastases (fig 3). In both plots PCI is associated with a significant delay to the time of relapse ($P = 0.02$) for cerebral metastases and for all neurologic relapses ($P = 0.01$). Studies of survival data adjusted for major prognostic variables showed no significant advantage for patients who had received PCI (fig 4). The median duration of survival for CR patients not receiving PCI was 13.5 months and the median survival for CR patients receiving PCI was 17.0 months. Comparison of the curves by Wilcoxon analysis indicates a statistically insignificant difference ($P = 0.07$) and comparison by the Mantel-Haenszel method shows a value of $P = 0.18$. The imbalance between PCI+ and PCI- patients in percentage of limited disease and thus the adjustment for limited versus extensive disease make the survival difference even further removed from achieving statistical significance ($P = 0.436$).

DISCUSSION

Postmortem studies from the 1960s and early 1970s reported cerebral metastases in 28%–55% of patients with SCLC (16). Through the 1970s, combination chemotherapy became a major component of the initial management of SCLC because of its ability to produce major responses and substantially improved survival in most patients, irrespective of disease extent. However, as predicted early in the 1970s, improved survival has been accompanied by an increased relative risk of CNS relapse, with some series suggesting that the cumulative CNS metastasis rate after effective therapy may be as high as 80% at 2 years (2,8,17). Thus, despite the therapeutic progress most patients ultimately relapse, with the possibility of treatment failure occurring within sanctuary sites (1,2,18,19). Relapses in nonsanctuary sites can be considered a consequence of systemic drug resistance and better control will require more effective agents or better treatment modalities. Sanctuary site relapses, however, may potentially be controlled in part by presently available, effective, local measures.

Although some chemotherapeutic agents which cross the blood-brain barrier, such as lomustine and procarbazine, may be active in SCLC, they are not known to favorably influence the incidence of CNS relapses (1,2). In addition, high-dose methotrexate has been tested and shown to be ineffective in preventing CNS metastases (20–22). Many combination chemotherapeutic protocols incorporating one or more of the above agents but without PCI appear to have a neurologic relapse rate similar to that without these agents (1,2,9,23). With the current failure of systemic chemotherapy to provide adequate control of micrometastases in sanctuary sites, it is appropriate that the greatest attention be directed towards PCI for the control of cerebral relapse. There is a well-known relationship between tumor size and radiosensitivity so that the optimal time for achieving sterilization of CNS disease is in the irradiation of micrometastases. The radiation sensitivity of SCLC is well-ap-

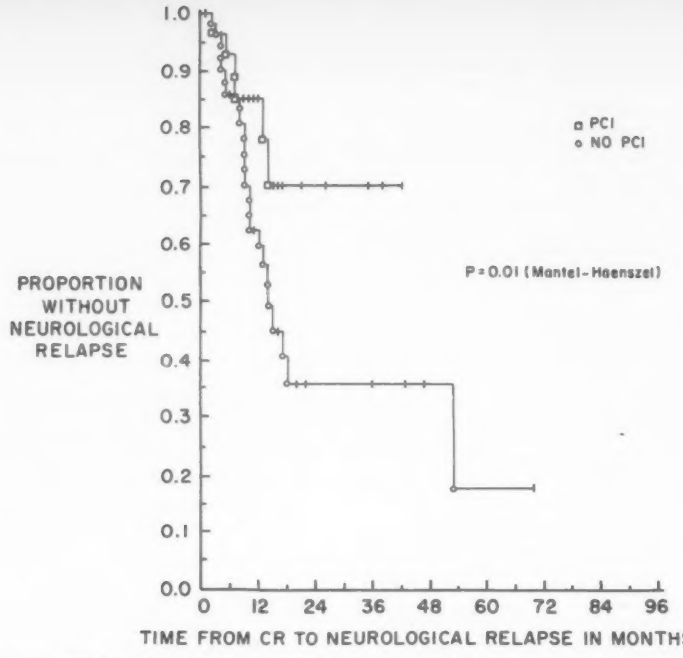

FIGURE 2.—Actuarial curve illustrating time from CR to any neurologic relapse, according to whether PCI was administered. Minimum follow-up = 10 mos.

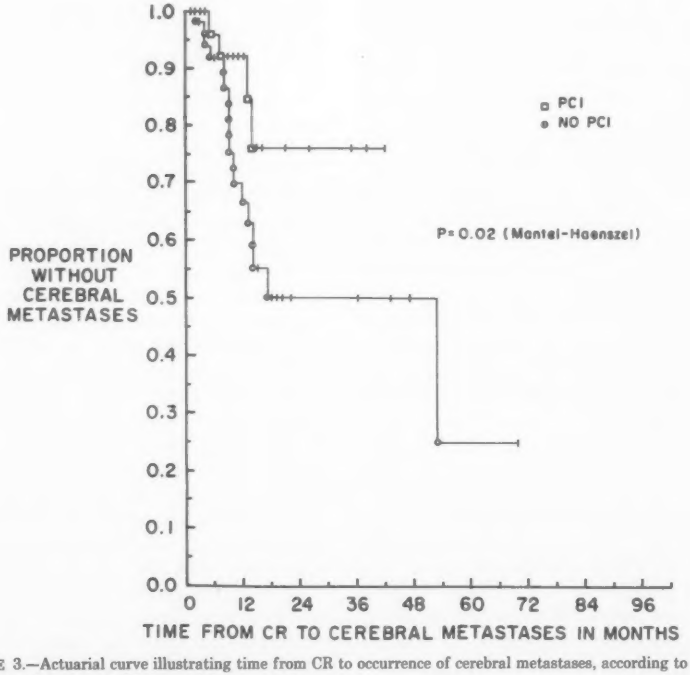

FIGURE 3.—Actuarial curve illustrating time from CR to occurrence of cerebral metastases, according to whether PCI was administered. Minimum follow-up = 10 mos.

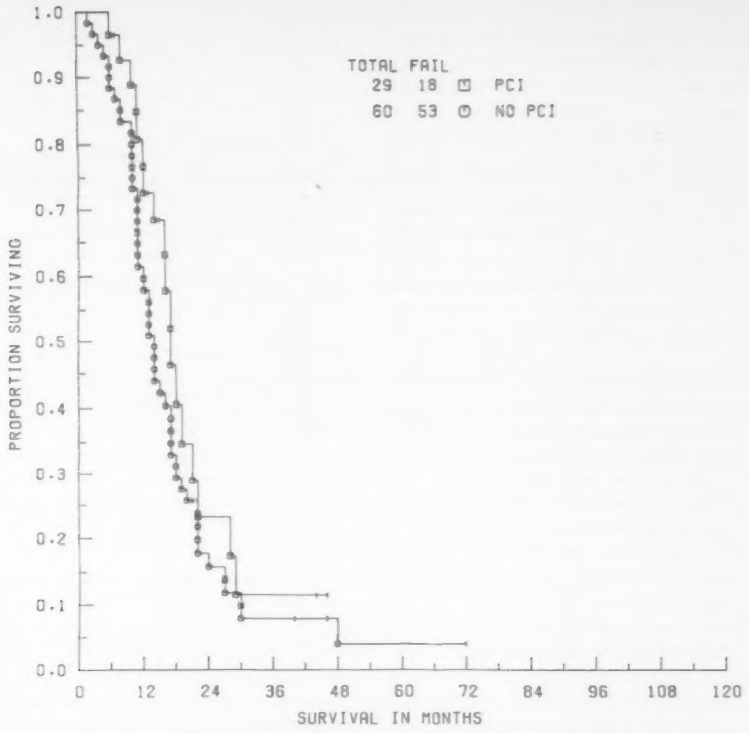

FIGURE 4.—Actuarial survival curve illustrating survival, according to whether PCI was administered, among patients achieving CR. $P = 0.07$ by Wilcoxon test, $P = 0.18$ by Mantel-Haenszel test after adjusting for limited vs extensive disease ($P = 0.436$).

preciated and, in the circumstance of low tumor burden and adequate dose, tumor sterilization can be expected (4).

In recent years it has been well-established that PCI reduces the incidence of clinically detectable cerebral metastases to about 3%–12%, but a concomitant improvement in survival has not been demonstrated (1,3–6,8,9). In addition, the incidence of meningeal carcinomatosis appears unaffected and may be another consequence of improved survival and tumor escape within sanctuary sites (12,13,17,23–26). One of the main reasons that PCI cannot be shown to affect survival is that most patients have relapses in systemic nonsanctuary sites; this is the major limiting factor to survival for the entire population (1,3). With the development of more effective systemic therapy it will be possible to show a survival benefit in relatively small studies. However, with the current limited long-term survival and the current fraction of potential CNS sanctuary site relapses, improvement in survival due to PCI is not easily demonstrated in present studies (1,3). There is a reduction in morbidity from neurologic relapses for some patients, particularly those who have any prolongation of survival, but this must be considered against cost, time, and eventual CNS relapse in many patients if PCI is given to all patients. It may be preferable to identify and treat only a subpopulation of patients who are most likely to benefit from the application of PCI. In most reports, the remission status of patients receiving PCI and the subsequent development of neurologic relapses have been given surprisingly little attention, even in well-randomized studies. It has thus not been possible to fully determine if a difference occurred between PCI+ and PCI- groups relative to the level of systemic response.

However, there is now an increasing body of evidence which suggests that complete responders are the group of patients deriving the greatest, if not the only, benefit from PCI (26–28). Levitt et al (27), in a study which utilized thoracic and whole-brain irradiation (3000 R) before the start of combination chemotherapy, found PCI ineffective in patients who did not achieve CR. Of 59 patients, five developed CNS metastases, none of whom had achieved CR. Our initial analysis and report of the alternating ACE/COMP study suggested that the patients with CR benefited from PCI, while those who did not achieve CR did not have sufficiently long survival to derive this benefit (26). Bunn et al (26) reviewed 93 SCLC patients after PCI was given (2000–2400 rad) to respond-

ers (CR and PR) at 12 or 24 weeks from the start of combination chemotherapy. They concurred and reinforced our initial findings, that patients achieving CR benefited from PCI, and that there was a significant reduction in the frequency of cerebral metastases in patients with CR. In addition, they found a trend for improved survival in CR patients receiving PCI.

Our current study was directed at defining the value of PCI to CR patients, based on the concept that maximum benefits could be expected in this group because of the potential for prolonged survival and the increasing likelihood of clinical CNS metastases for up to 2 years. Our findings confirm our earlier preliminary conclusions and are consistent with those of Levitt et al (27) and Bunn et al (26). These indicate a significant delay in the occurrence of cerebral metastases in complete responders given PCI at CR, and most particularly show a significant reduction in cerebral relapse as the initial or sole site of relapse. These findings are also compatible with the concept that with improved systemic treatment PCI given to patients with CR may ultimately increase the number of long-term survivors. Clearly, PCI can eliminate cerebral micrometastases. For some patients this may be the only site of residual disease, so that even though it is not reflected in statistically significant survival curves, some of the patients with CR will have improved survival, and possibly cure, with the addition of PCI. One may speculate that the patients with CNS-only disease at the time of death may have been long-term survivors had they received PCI.

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Phase I Trial and Pharmacokinetics of Acivicin Administered by 72-Hour Infusion[1,2,3]

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Acivicin, an L-glutamine antagonist, was administered to 37 evaluable patients with refractory advanced solid tumors in a phase I trial. A total of 67 evaluable 72-hr iv infusions were given at 3- to 4-week intervals. Doses ranged from 3.0 to 90 mg/m²/course. Reversible CNS toxicity was dose-limiting and included lethargy, somnolence, anxiety, hallucinations, and paranoid psychoses. Four of five patients experienced unacceptable CNS toxicity at 90 mg/m². Three of eight patients experienced reversible diaphoresis and chills without fever at 75 mg/m², and two had dizziness and ataxia. Hematopoietic toxicity, nausea, emesis, and diarrhea were mild and dose-related. One patient developed a blue-green discoloration of the infusion arm. Serial plasma and urine specimens from 13 patients were assayed for acivicin using a microbiologic method. Peak plasma levels at the end of the 72-hr infusions correlated with dose and ranged from 0.09 to 1.10 μg/ml. When data from six patients were fitted to a two-compartment open model, alpha-half-life ranged from 1.1 to 63 mins, while beta-half-life ranged from 338 to 629 mins. Renal clearance ranged from 6 to 24 ml/min, and nonrenal clearance accounted for 58%–83% of the total drug clearance. CNS toxicity correlated with plasma acivicin levels which exceeded 0.9 μg/ml for > 16 hrs, but not with peak plasma levels or with the integrals of the concentration × time curves. Minor responses were seen in one patient with melanoma, in one with epidermoid pulmonary carcinoma, and in two with colon carcinoma. A starting dose of 60 mg/m²/course was recommended for phase II trials, with possible escalation to 75 mg/m² in the second course if the drug was well-tolerated. [Cancer Treat Rep 67:683–692, 1983]

Acivicin is an L-erythro amino acid isolated from the fermentation broth of *Streptomyces sviceus* (1,2). Preclinical studies reveal significant increases in lifespan induced by acivicin in the murine L1210 ascitic leukemia model (3). There is also activity in P388 murine ascitic leukemia and in human breast and colon xenografts in athymic nude mice (4). This activity was responsible for selection of acivicin as a promising agent for phase I clinical trials (5).

Acivicin is an S phase-specific antimetabolite (6) which undergoes rapid urinary excretion in the mouse (7). These factors may explain the significant schedule dependency of acivicin in the L1210 system. Maximum benefit is achieved by ip injections every 3 hrs for 24 hrs on the Day-1, -5, and -9 treatment schedule (8). Consequently, we sought to establish the maximum tolerated dose of acivicin using a 72-hr continuous iv infusion schedule in man. Pharmacokinetic investigations were performed concomitantly to identify significant distributional or excretory factors in the clinical setting. CNS toxicity was dose-limiting and correlated with exposure to plasma acivicin levels > 0.9 μg/ml for > 16 hrs.

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MATERIALS AND METHODS

Eligibility criteria and evaluation methods were described in one of our previous phase I trial reports (9).

Acivicin was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, in sterile vials containing 25 mg of lyophilized drug. Each vial was aseptically reconstituted with sodium chloride for injection, USP (without preservative). The daily dose was administered in 1000 ml of sodium chloride for injection, USP, over a 24-hr period by IVAC pump. This infusion was repeated three times over a total period of 72 hrs. Based on a toxic dose low of 10 mg/m²/course on both daily × 5 and daily × 10 schedules in beagle dogs (8), the initial clinical dose level was 3.0 mg/m²/course. Escalations were planned to doses of 6, 9, 15, 21, 27, 36, 48, 60, 75, and 90 mg/m²/course according to a modified Fibonacci scheme. After communication with other investigators who were studying acivicin, we modified our dose escalation scheme to omit the levels of 21, 27, and 48 mg/m²/course. Courses were repeated every 21 days unless delayed for unrelated clinical reasons. Dose escalations above the entry level for individual patients were prohibited.

Peripheral venous blood samples were drawn at 24, 48, and 72 hrs during the infusion period from one to three patients at each dose level. Heparinized plasma was prepared and frozen for analysis of intact acivicin. At dose levels > 36 mg/m²/course, samples were also collected at 0, 15, 20, 45, 60, 90, 120, 240, 480, 720, 1080, and 1440 mins after infusion, if possible. Urine was collected every 6 hrs during and following the infusion period, total volumes were measured, and aliquots were frozen for analysis.

Acivicin concentrations in plasma and urine of treated patients were assayed by a microbiologic disc-tray method using *Bacillus subtilis* UC-902 cultivated in a completely synthetic medium (1,10). Modifications of this procedure for assay of plasma and urine were previously published (7). Whenever possible, pretreatment plasma and urine specimens were obtained and standard curves were prepared using each patient's own fluids. Otherwise, plasma or urine from normal human subjects was used in the preparation of standards. The assay had a sensitivity of about 0.06 μg/ml in plasma; the sensitivity was about 0.6 μg/ml in urine due to background interfering substances. The coefficient of variation for the assay was 7%.

To determine the specificity of the microbiologic growth-inhibition assay, bioautographic studies were carried out on urine specimens from four selected patients as previously described (7). Briefly, aliquots of urine were chromatographed on silica gel in thin-layer plates using four different solvent systems. The plates were dried after development, placed in contact with agar trays seeded with the assay microorganism, and then removed after sufficient time for elution of the separated materials from the gel into the agar. The trays were then incubated and the resulting zones of growth inhibition were used in calculating R_f values, which were matched with those of acivicin standards.

Plasma concentration data were fitted to a two-compartment open model, and estimates of model parameters were obtained by previously published methods (11). The measured urinary drug concentration over the third 24-hr period of the 72-hr infusion, the urine flow rate, and the plateau serum drug concentration were used to calculate renal and nonrenal clearances. The concentration × time curves for other infusion schedules were simulated by computing

$$C = (K_0/V_c)\left[\left(\frac{(K_{21}-\alpha)(1-\exp(\alpha T))}{\alpha(\alpha-\beta)}\right)\left(\frac{1-\exp(-n\alpha\tau)}{1-\exp(-\alpha\tau)}\right)\exp(-\alpha t)\right.$$

$$\left.+\left(\frac{\beta-K_{21})(1-\exp(\beta T))}{\beta(\alpha-\beta)}\right)\left(\frac{1-\exp(-n\beta\tau)}{1-\exp(-\beta\tau)}\right)\exp(-\beta t)\right]$$

for appropriate time points t. The infusion rate constant K_0 was calculated for each infusion by:

$$K_0 = D_0/T_i,$$

where T_i was the infusion duration. In this computation, τ was the interval between the start of each of n infusions; $t = 0$ at the start of each infusion; $T = t$ for $t \leq T_i$; and $T = T_i$ for $t > T_i$ (12,13).

RESULTS

Thirty-nine eligible patients were entered in this phase I trial. A total of 75 courses of acivicin were administered, and 67 of these were fully evaluable for toxicity. Two patients who developed acute psychotic reactions at 90 mg/m²/course refused to complete the full 3-day infusion and did not allow collection of blood for subsequent evaluation of toxicity. Six other patients at various dose levels did not receive adequate laboratory follow-up in their final treatment course. All patients had received prior chemotherapy, and nine had also received prior radiation therapy. Twenty-two patients had an Eastern Cooperative Oncology Group performance status of 0 or 1, and 17 had an Eastern Cooperative Oncology Group performance status of 2 or 3. Patients had advanced solid tumors of the types shown in table 1.

CNS toxicity proved to be dose-limiting on this treatment schedule of acivicin. Of 11 patients treated at 75 mg/m²/course, one experienced dizziness and one had ataxia and blurred vision. At 90 mg/m²/course, CNS toxicity was intolerable. One patient developed tinnitis and high-frequency hearing loss after 1 day of infusion, and on the second day she developed acute paranoid

psychotic tendencies; she left the hospital against medical advice. She developed difficulty with ambulation at home. These symptoms resolved after 3 days. A second patient developed hallucinations and paranoid psychotic reaction lasting 1 week following a 72-hr infusion. A third patient developed extreme lethargy on the third day of the infusion. This improved within hours after the infusion was discontinued, but the patient developed visual and auditory hallucinations on the subsequent 2 days. A fourth patient developed lightheadedness following his infusion, became extremely anxious, and required mild sedation for the subsequent week. Only one of five patients tolerated this dose without CNS side effects. None of these patients had a history of overt psychiatric problems or of sedative, antidepressant, tranquilizer, or other psychoactive agent use.

TABLE 1.—Types of tumor in patients treated with acivicin

Type	No. of patients
Colon	11
Breast	9
Gastric	3
Head and neck	3
Lung	2
Renal	2
Melanoma	2
Ovary	2
Sarcoma	1
Lymphoma	1
Bladder	1
Testicular	1
Adenocarcinoma of unknown origin	1

Three patients given 75 mg/m²/course developed an unusual drug-related toxic effect comprising diaphoresis followed by chills without pyrexia. This reaction was not observed at the higher dose (90 mg/m²/course). The reaction occurred predictably at 46–55 hrs after starting the infusion and subsided within a few hours after termination of drug administration. Their acivicin was from the same lot as that received by all other patients. The reaction recurred in two of the three patients during the subsequent course of treatment with acivicin. Pupillary diameter and reflexes were normal, there was no change in cardiac rate or rhythm, and there was no postural hypotension. Valsalva maneuver produced appropriate bradycardia with compensatory tachycardia. Diaphoresis was general in distribution, and there was no excessive salivation. The remainder of the neurologic examination was unremarkable. Serum glucose was normal in one patient during the episode. Cultures of iv administration equipment, blood, urine, and other sites were uniformly negative. One patient was given atropine at a dose of 0.4 mg sc during the episode. This seemed to improve her symptoms, but because the drug infusion was terminated it is impossible to determine whether atropine truly antagonized the syndrome. During a subsequent course at 60 mg/m², a urine collection over the last 24 hrs of the infusion period showed amino acid excretion within the normal ranges.

Other toxic effects were generally mild and are shown in table 2. Hematologic toxicity was dose-related. The one patient who experienced grade 4 thrombocytopenia had been treated for breast cancer with numerous agents including doxorubicin, vincristine, dibr-

TABLE 2.—Non-CNS toxicity of acivicin by dose, type, and grade

Dose (mg/m²/course)	No. of evaluable patients	Grade*	No. of patients with—					
			Leukopenia	Thrombocytopenia	Emesis	Diarrhea	Stomatitis	Malaise/anorexia
3	3	1	1	0	0	1	0	0
6	3	1	1	0	1	0	0	0
9	3	1	2	0	0	0	0	0
15	3	1	0	0	0	1	0	0
36	6	1	2	0	0	0	0	2
		2	0	0	0	0	0	0
		3	0	0	0	0	0	0
		4	0	1	0	0	0	0
60	3	1	1	0	1	1	0	0
		2	0	0	1	0	0	0
75	11	1	3	1	1	1	0	1
		2	4	0	3	0	0	0
		3	0	1	1	0	0	0
90	5	1	1	0	2	0	0	0

*Grade 1 = mild; 2 = moderate; 3 = severe; 4 = life-threatening.

modulcitol, hexamethylmelamine, methotrexate (both standard and high-dose, with rescue), 5-FU, cyclophosphamide, bleomycin, tamoxifen, melphalan, mitomycin, halotestin, and prednisone over 4.5 years. Her platelet count nadir of 22,000/mm³ occurred 8 days after 36 mg/m² of acivicin and recovered to normal levels by Day 14. Milder thrombocytopenia was also seen in several patients who received 75 mg/m²/course of acivicin and was sometimes accompanied by mild leukopenia. Nausea and emesis were dose-related but usually controllable. Sporadic patients had diarrhea, anorexia, and malaise. One patient developed a bluish-green discoloration of the arm which received the drug.

There were no objective complete or partial therapeutic responses. Minor responses, including stable measurable disease for > 9 weeks, improved laboratory values (alkaline phosphatase), and improved performance status occurred at 36 mg/m²/course in one patient with epidermoid pulmonary carcinoma and at 75 mg/m²/course in two patients with colon carcinoma and one patient with melanoma. The last response persisted for > 54 weeks.

Because the microbiologic assay has a sensitivity limit of 0.06 μg/ml, postinfusion pharmacokinetic studies could not be performed on patients who received acivicin at < 60 mg/m²/course. Plasma acivicin concentrations measured during the infusions and urinary recoveries are shown in table 3. Most patients had achieved steady-state plateau levels by 24 or 48 hrs. Plasma levels at 72 hrs were highly correlated with the acivicin dose ($r = 0.87$; $P < 0.01$).

Figure 1 presents plasma decay curves obtained by fitting a two-compartment open model to the concentration × time data from three patients at higher dose levels. The two-compartment model was fitted to the data with correlation coefficients ranging from 0.962 to 0.991. Pharmacokinetic parameters related to distribution are presented in table 4. Zero-time intercepts reflect the hypothetic central compartment (A) and peripheral compartment (B) drug concentrations for the bolus case, as calculated from the fitted postinfusion plasma decay curves. Analysis of data from Patients 8 and 11 yielded unusually low and high values (respectively) for the zero-time concentrations in the central compartment. These values led to high and low values (respectively) for the normalized volume of the central compartment as well as for the associated intercompartmental first-order transfer rate constants. These calculated parameters may be inaccurate due to inadequate data for correct estimation of the central compartment intercepts in these patients. Consequently, the mean central compartment volume of distribution was calculated from the other four patient values. This hypothetic volume of distribution, 0.20 L/kg, is approximately equivalent to the expected inulin space (plasma plus interstitial-lymphatic volume) in normal man (13).

The distributional phase half-lives (table 5) ranged from 1.1 to 63.3 mins, possibly due to a rapid distributional phase which was not accurately quantitated by the sampling intervals immediately after infusion. The elimination phase half-lives ranged from 338 to 629 mins (5.6–10.5 hrs). Based on the four patients whose calculated parameters seemed to be reliable, the mean alpha-half-life was 24 mins and the mean beta-half-life was 493 mins. Neither the end-infusion drug burden nor the area under the concentration time curve correlated with dose. Urinary drug recoveries ranged from 30% to 41% of the dose. Based on the third-day drug recovery in urine, the steady-state renal clearance averaged 17 ml/min. Nonrenal clearance exceeded renal clearance in all patients and accounted for 58%–83% of the total drug clearance.

DISCUSSION

Biochemical, pharmacokinetic, and cytokinetic actions of acivicin must be considered in the selection of an appropriate schedule for phase II trials. The in vitro inhibition of L1210 cell growth by acivicin is antagonized by L-glutamine (6) or by combinations of cytosine and guanosine ribonucleosides or deoxyribonucleosides (14). Cytidine triphosphate synthetase, a glutamine-dependent amidotransferase, is strongly inhibited by acivicin. Elevation of cellular UTP pools with concomitant decreases in CTP and GTP pools indicates that the cytotoxic effects of acivicin may be mediated by inhibition of cytidine triphosphate synthetase as well as by inhibition of xanthosine monophosphate aminase, another glutamine-dependent enzyme (14). Acivicin also inhibits carbamoyl phosphate synthetase (15) and thus decreases early de novo pyrimidine synthesis. These biochemical effects underlie the cytokinetic action of acivicin, cell cycle arrest in G_1 and S phases (6). Such agents are best administered at constant levels over periods which are tolerated by the normal marrow stem cell pool (16,17).

The 72-hr infusion of acivicin at doses of 60, 75, and 90 mg/m²/course achieved steady-state plasma drug levels of 0.46–1.10 μg/ml. The number of patients studied at each dose level was too low for meaningful comparisons between dose levels or for statistical correlation with toxicity or pathophysiology. The data do permit conclusions regarding the pharmacokinetics of acivicin in man.

Plasma acivicin levels fitted a two-compartment open pharmacokinetic model, although the distribution phase was variable among patients. This observation supports the contention by Gibaldi and Perrier (12) that ". . . following infusion the ability to discern the two-compart-

TABLE 3.—Plasma concentration and urinary recovery data in patients receiving acivicin as a 72-hr infusion

Patient No.	Sex	Dose level (mg/m²/course)	Plasma concentration (μg/ml) at times during infusion (hrs)			Urinary recovery (mg) at collection intervals (hrs)				Total urinary recovery * (% of total dose)
						During infusion			After infusion	
			24	48	72	0-24	24-48	48-72	0-24	
1	F	3	—†	—†	—†	0.3	—‡	—‡	—§	§
2	F	9	0.22	0.25	0.086	—‡	—‡	—‡	—§	§
3	M	15	0.14	0.16	0.17	0.3	0.5	—‡	—§	§
4	M	15	0.29	0.14	0.15	3.4	6.1	5.3	—§	§
5	M	36	0.13	0.12	0.13	3.2	5.9	6.1	—§	§
6	M	64.2¶	0.38	0.36	0.47	4.5	13.7	—‡	—§	§
7	F	60	0.38	—‡	—‡	5.8	—‡	—‡	—§	§
8	M	75	—‡	0.74**	0.89	3.8	22.8	13.3	4.2	31
9	F	75	0.58	0.58	0.57	6.4	13.7	16.9	6.0	33
10	M	75	0.40	0.44	0.46	7.1	14.8	16.0	7.4	30
11	M	90	3.4	2.6	1.1	13.8	23.0	24.6	8.4	42
12	F	90	0.52	0.58	0.56	13.2	19.4	18.2	5.6	31
13	M	90	0.75	0.84	††	5.7	12.7	—‡	—§	§

*Through 24 hrs after infusion.
†Plasma concentration below limit of assay sensitivity (about 0.06 μg/ml).
‡Sample not taken.
§Not determined because of incomplete urine collection.
¶High dose given due to erroneous calculation of surface area.
**Sample drawn at 53 hrs.
††Infusion discontinued because of CNS toxicity.

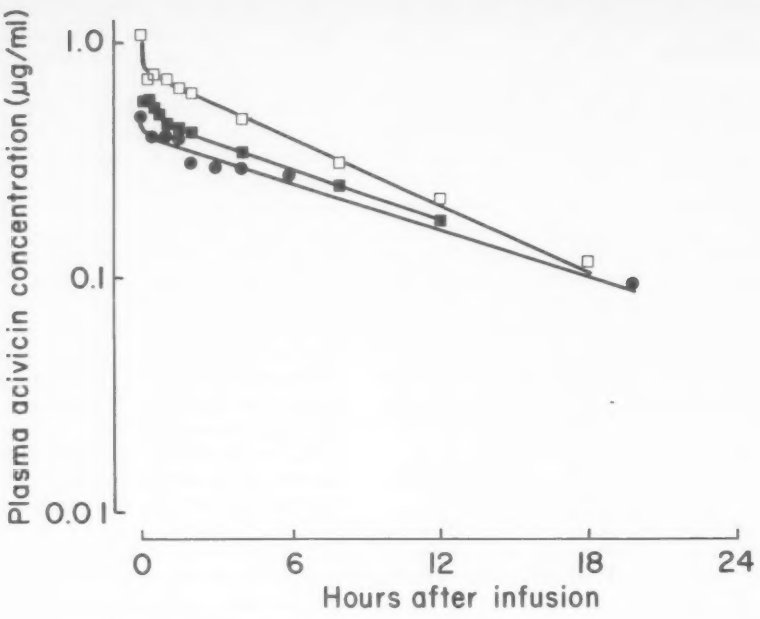

FIGURE 1.—Postinfusion plasma acivicin levels and fitted curves in patients who received 72-hr infusions of 64.2 mg/m² (●), 75 mg/m² (■), and 90 mg/m² (□).

TABLE 4.—Distributional pharmacokinetic parameters of acivicin

Patient No.	Dose level (mg/m²/course)	Peak serum level (µg/ml)	Zero-time intercepts A′ (µg/ml)	Zero-time intercepts B′ (µg/ml)	Normalized V_c (L/kg)	Micro rate constants ($min^{-1} \times 10^2$) K_{12}	K_{21}	K_{10}
6	64.2	0.47	8.2	1.9	0.17	1.5	5.5	5.1
8	75	0.89	0.21	4.1	0.46	0.04	0.1	1.2
9	75	0.57	10.0	2.7	0.15	1.0	5.0	5.0
10	75	0.46	5.5	2.4	0.22	1.8	10.2	4.0
11	90	1.10	883	6.0	0.002	42.3	6.0	18.7
12	90	0.56	5.8	4.8	0.26	4.2	38.7	4.4

ment characteristics of a drug is usually decreased." Future studies should collect multiple samples during the first hour following infusion to achieve better definition of the distribution phase. Acivicin clearance from plasma had an average half-life of 8.5 hrs and was dominated by nonrenal clearance. Presumably this reflected drug metabolism since gastrointestinal excretion has not been identified as a major acivicin excretion pathway. Overall, urinary drug recovery averaged 35% of the administered dose and was highest at high doses. Renal clearance was much less than the creatinine clearance, indicating that tubular reabsorption of acivicin occurs in man. This suggests significant species differences in acivicin pharmacokinetics and metabolism, since acivicin renal excretion exceeds the creatinine clearance in the mouse and 53%–82% of the drug is excreted in intact form by that species (7).

The structure of acivicin suggests that the proximal tubular amino acid absorptive processes may be responsible for active reabsorption of the drug. Acivicin uses cell membrane amino acid uptake mechanisms in vitro (18). Such mechanisms may be saturable by high drug levels in the glomerular filtrate. We found only slight evidence of dose-related increases in acivicin excretion. We also found excretion-phase half-lives which were comparable to those reported for other schedules (19). Patients with defects in amino acid reabsorptive mechanisms might clear acivicin more rapidly than did our

TABLE 5.—Pharmacokinetic clearance parameters of acivicin

Patient No.	Dose level (mg/m²/course)	α-half-life (mins)	β-half-life (mins)	D* (mg)	Area under the curve (mg · min/ml)	Urinary recovery (%)	Clearance (ml × min⁻¹)		
							Total	Renal	Nonrenal
6	64.2	28.7	595	22.3	2.00	—†	68	20	48
8	75	63.3	629	29.9	3.74	31	38	10	28
9	75	37.3	518	17.9	2.54	33	51	21	30
10	75	22.5	520	23.7	1.97	30	76	24	52
11	90	1.1	382	15.0	4.75	42	35	6	29
12	90	8.4	338	15.2	2.40	41	57	23	34
Mean	—	—	—	—	—	35	51	17	34

*D = end-infusion drug burden.
†Collection was inadvertently lost.

patients. Early clinical trials of cycloleucine, a nonmetabolizable cyclic α-amino acid, demonstrated CNS toxicity in a significant fraction of patients (20). This agent induces marked, selective aminoaciduria which is similar to hereditary cystinuria but which is reversible (21). Since acivicin also induced CNS toxicity in our study and appeared to interact with tubular amino acid reabsorptive mechanisms, we studied urinary amino acid excretion in one patient. No evidence for selective aminoaciduria was seen.

Acivicin distributes to the brain in the mouse (8) and to the cerebrospinal fluid in monkeys (22). Clinical dose-limiting CNS toxicity implies that this is also true in man. The mechanism of action of acivicin in the CNS remains unclear. We observed generalized diaphoresis followed by chills without fever in three patients, and the effect was reproduced in two of these. We hypothesized that a general parasympathomimetic effect might be involved, but we found no clinical evidence supporting this mechanism in one patient who was examined with this in mind. Atropine apparently relieved the symptoms but was not clearly antidotal. We cannot conclude that the diaphoresis-chills symptom complex was mediated by the same mechanism that caused mental changes at high doses. Patients who exhibited the latter did not experience diaphoresis or chills associated with drug infusion. Until the mechanism of CNS toxicity is clarified, treatment of acivicin effects with psychoactive drugs is contraindicated.

Major CNS toxicity in our study did not occur during an infusion of doses $\leq$ 75 mg/m² but was very common at 90 mg/m². We simulated the plasma concentration ×

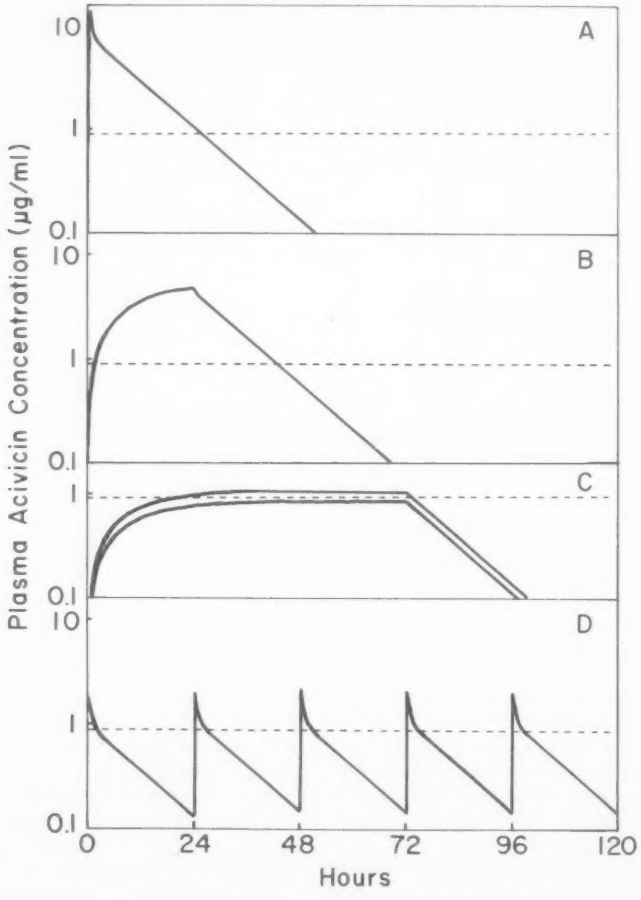

FIGURE 2.—Simulations of (A) 15-min infusion at 120 mg/m², (B) 24-hr infusion at 160 mg/m², (C) 72-hr infusions at 75 and 90 mg/m², and (D) daily × 5 bolus injections at 75 mg/m².

TABLE 6.—Calculated pharmacokinetic parameters and toxicity of 4 acivicin schedules

Schedule	Total dose (mg/m²/course)	Peak level (μg/ml)	Area under the curve (mg · min/ml)	Duration > 0.9 μg/ml (hr)	Toxicity	Ref No.
Bolus	120	13.85	5.86	26.6	CNS	(23)
24-hr infusion	160	4.66	7.69	41.3	CNS	(19)
72-hr infusion	90	0.99	4.26	46.1	CNS	—
	75	0.82	3.55	0	Rare CNS	—
Daily × 5	75	2.05	3.56	16.9	Hematologic + mild CNS	(24,25)
	60	1.64	2.85	9.7	Hematologic	(24,25)

time curves which might be expected from four different schedules of acivicin administration. Figure 2 illustrates the theoretic plasma acivicin concentration × time curves which resulted from the simulation. Peak levels, integrals of the simulated concentration × time curves, and durations of plasma concentration > 0.9 μg/ml are shown in table 6. These values and the graphic simulations of figure 2 were calculated based on average patient parameters in our study (weight = 71.4 kg; surface area = 1.865 m²; $\alpha = 0.0286\ min^{-1}$; $\beta = 0.00141\ min^{-1}$; $K_{21} = 0.01485\ min^{-1}$; and V_c = 14.28 L). There is no correlation between peak plasma acivicin level and CNS toxicity. Although a concentration × time integral > 4 mg · min/ml was usually associated with CNS toxicity, the latter is also reported in the daily × 5 schedule at 75 mg/m² (24,25). That schedule achieved the same concentration × time integral as did a 72-hr infusion of the same dose. All regimens which achieved a simulated plasma concentration > 0.9 μg/ml for > 16 hrs were associated with CNS toxicity. We hypothesize that the CNS toxicity of acivicin is related either to the duration of exposure to suprathreshold drug levels or to the integral of the concentration × time curve rather than to the magnitude of the peak level. If the former is the case, the plasma threshold for CNS toxicity is of the order of 0.9 μg/ml and the critical duration is approximately 16 hrs.

Clinical benefit was achieved in two patients with colon carcinoma, one patient with epidermoid lung carcinoma, and one patient with melanoma. Phase II trials are indicated in these diseases and in tumors which may be sensitive to S phase-specific antimetabolites. An initial dose of 60 mg/m²/course, with escalation to 75 mg/m²/course as tolerated, is recommended for the 72-hr infusion regimen.

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Phase I Trial of Homoharringtonine Administered as a 5-Day Continuous Infusion[1,2,3]

Craig J. Coonley, Raymond P. Warrell, Jr,* and Charles W. Young[4,5]

Homoharringtonine (HHT) has substantial cytotoxic activity against cell lines of experimental tumors. Moreover, the drug has been used extensively in the People's Republic of China for the treatment of patients with acute leukemia. Since rapid injections of HHT can produce serious hypotension and cardiac arrhythmias, we evaluated HHT administered as a continuous infusion for 5 days in patients with advanced cancer. Thirty-one patients were treated with HHT at doses which ranged from 0.2 to 3.75 mg/m²/day. Myelosuppression (both leukopenia and thrombocytopenia) was the dose-limiting toxic reaction. Platelet recovery was delayed in five patients; the median time to platelet nadir was 31 days in patients treated at the highest dose level. Sinus tachycardia occurred frequently, but the relation of this effect to drug administration was uncertain. Serious cardiac arrhythmias and hypotension were not observed using this schedule. We conclude that HHT can be safely administered as a continuous infusion. A daily dose of 3.25 mg/m² × 5 days is recommended for phase II studies. [Cancer Treat Rep 67:693–696, 1983]

Homoharringtonine (HHT) (fig 1) is a cephalotaxine alkaloid whose antitumor effects have long been recognized in Chinese folk medicine (1). Preclinical screening in this country revealed substantial cytotoxic activity against L1210 and P388 leukemias as well as colon 38 and CD8F51 mammary carcinomas. The drug was inactive against Lewis lung carcinoma and xenografts of human lung, colon, and breast cancers implanted in nude mice (2). In preclinical tests, HHT produced dose-related toxic effects on bone marrow, cardiac, lymphatic, gastrointestinal, hepatic, and renal tissues of both mice and dogs (2).

The Chinese have successfully used both harringtonine and HHT alone and in combination with other drugs for the treatment of patients with acute nonlymphocytic leukemia (3–5). Myelosuppression was recorded as the predominant toxic reaction but tachyarrhythmias, ECG changes, and nausea were also observed. Chinese investigators have recommended a slow iv infusion schedule (3,4) since bolus administration appeared to increase the incidence of acute toxic reactions without increasing the therapeutic benefit (3). To avoid the acute autonomic toxicity, we evaluated the biologic effects of HHT administered by continuous infusion for 5 days in a series of patients with advanced cancer.

PATIENTS AND METHODS

Patients were eligible for this study if they had failed conventional therapy. They were required to have a life expectancy of > 6 weeks and adequate renal and hepatic functions (defined as serum creatinine and total serum bilirubin concentrations ≤ 1.5 mg/dl). All patients had normal blood cell counts (ie, total wbc count ≥ 4000 cells/mm³ and platelet count $\geq 150{,}000$ cells/mm³). Patients with cardiac arrhythmias or a history of myocardial infarction within the preceding 12 months were excluded. The absence of bidimensionally measurable disease or prior treatment with anthracyclines did not exclude entry.

All patients had a complete history and physical examination. Additional procedures were performed as

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[3]Homoharringtonine was supplied by the Division of Cancer Treatment, National Cancer Institute.

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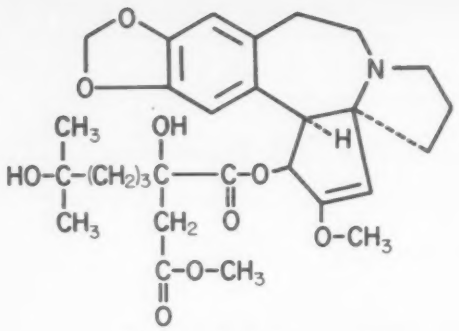

FIGURE 1.—Chemical structure of HHT.

needed to define the extent of disease. Cbcs were determined daily during the infusion and twice weekly for 4 weeks thereafter. Serum concentrations of electrolytes, BUN, and glucose were measured on Days 1 and 5, and a 12-channel biochemical screening profile was measured on Days 1 and 5 and every 2 weeks thereafter for 4 weeks. For all patients, ECG were performed and vital signs were measured as a baseline and then daily during the drug infusion. At the two highest dose levels, orthostatic pulse and blood pressure determinations were recorded four times/day during each infusion. After case entry was completed at the three lowest dose levels, dose escalation was not allowed in the same individual.

The prescribed daily dose of HHT was diluted in 1 L of 5% dextrose solution and administered with an infusion pump over 24 hours. A single course of therapy consisted of one infusion for 5 days. The starting dose was 0.2 mg/m²/day. A modified Fibonacci sequence (6) was used to determine dose escalation. Written informed consent was obtained from all patients and the study was approved in advance by the Memorial Sloan-Kettering Cancer Center Institutional Review Board.

RESULTS

We administered 33 courses of HHT to 31 patients. The diagnoses and pretreatment characteristics of these patients are presented in table 1. Thirty of the 31 patients were fully evaluable, each having received at least one full course. One patient (at the 2.5-mg/m²/day level) required emergency radiotherapy shortly after completion of infusion and was considered inevaluable for hematologic toxicity. No significant antitumor effects were observed in our patients.

Hematologic Toxicity

Myelosuppression was the dose-limiting toxic effect (table 2). Five of six patients receiving HHT at 3.75 mg/m²/day experienced moderate to severe leukopenia or thrombocytopenia (table 2). At doses ≤ 3.25 mg/m²/day, only four of 24 patients experienced serious leukopenia (< 2000 cells/mm³) or thrombocytopenia (< 50,000 cells/mm³). There were no important differences regarding type or extent of prior treatment among patients at any dose level. One patient required hospitalization for fever related to severe leukopenia, and four patients required platelet transfusions for a platelet count < 20,000 cells/mm³. Drug-related leukopenia generally reached a nadir by Day 14, while the platelet count nadir was frequently not reached until Day 30. Six patients required rbc transfusions because of anemia.

Nonhematologic Toxicity

All 31 patients were evaluated for nonhematologic toxic effects (table 3). In general, HHT was well-tolerated. Serial blood pressure measurements did not reveal serious hypotension in any patient. Neither systolic blood pressure < 80 mm Hg nor diastolic blood pressure < 50 mm Hg was recorded during the trial. No infusion was discontinued because of low blood pressure and no patient received specific treatment for hypotension. Serial ECG and pulse determinations revealed sinus tachycardia of uncertain significance in 30 patients (96%). Although this incidence is high, 14 patients entered the trial with pre-existing sinus tachycardia. Moreover, tachycardia was often manifested as an isolated increase in pulse rate and it was generally not sustained throughout the infusion. The rate of tachy-

TABLE 1.—Characteristics of patients treated with HHT

Characteristic	No. of patients
Total treated	31
No. of treatment courses: 33	
Median age in yrs (range): 57 (23–71)	
Pretreatment performance status (Karnofsky)	
80–90	2
70–80	6
50–60	23
Prior radiation therapy	26
Prior chemotherapy	29
Patient diagnosis (type or site of cancer)	
Lung	10
Lymphoma	5
Kidney	4
Breast	2
Colon	2
Sarcoma	2
Esophagus	1
Head and neck	1
Unknown primary	1
Mycosis fungoides	1
Ovary	1
Stomach	1

TABLE 2.—Hematologic toxic effects of HHT administered by continuous infusion

Dose level (mg/m²/day)	No. of patients entered	No. of patients evaluable	Leukocytes				Platelets			
			Nadir*		Day		Nadir*		Day	
			Median	Range	Median	Range	Median	Range	Median	Range
0.2	3	3	6.2	5.7-6.7	2	2-22	288	132-290	6	3-14
0.4	3	3	7.0	4.2-12.3	5	4-6	485	108-523	5	2-12
0.7	3	3	6.8	3.3-7.4	5	4-5	233	205-607	5	1-12
1.5	5	5	5.6	1.4-8.4	9	4-18	249	106-342	6	3-14
2.5	7	6	4.2	1.1-6.7	10	3-25	118	66-468	21	5-31
3.25	6	6	4.4	1.2-6.6	15	14-19	180	6-218	29	23-33
3.75	6	6	2.7	1.1-9.3	17	10-29	19	9-229	31	14-33

* × 10^3 cells/mm^3.

TABLE 3.—Frequency of nonhematologic toxic reactions to HHT (n = 31)

Toxic reactions	No. (%) of patients
Tachycardia *	30 (96)
Nonspecific ST-T wave changes on ECG	10 (33)
Arrhythmia†	2 (6)
Nausea/vomiting	9/3 (30/10)
Alopecia	4 (12)

*Maximum pulse > 100/min.
†Premature ventricular contractions.

cardia was not dose-related. Isolated premature ventricular contractions were noted on routine ECG obtained in two patients. One patient who had experienced a myocardial infarction 5 years before the study developed a brief episode of angina 2 days after completing the drug infusion. He was not hypotensive, and myocardial infarction was ruled out.

Nausea appeared to be dose-related, but vomiting was infrequent. Four patients developed mild alopecia at the 3.75-mg/m² dose level. No patient developed renal or liver dysfunction as a result of this therapy. One patient developed phlebitis at the infusion site. Although accidental extravasation of small amounts of drug occurred in several patients, no local tissue damage was observed.

DISCUSSION

A principal aim of this study was to establish the maximally tolerable dose of HHT administered as a continuous infusion for 5 days. We found that myelosuppression was the dose-limiting toxic reaction in this schedule. Although daily doses of 2.5 and 3.25 mg/m² × 5 days were relatively well-tolerated, most patients who received 3.75 mg/m²/day × 5 developed serious hematologic toxic effects. The leukocyte nadir generally occurred within 2 weeks from the initiation of treatment, but the platelet nadir was frequently delayed beyond Day 30. Moreover, recovery of the platelet count to normal was quite prolonged in several patients.

We observed sinus tachycardia in almost all patients who did not have baseline tachycardia. This effect may have been drug-related although we noted no relationship between heart rate and dose of HHT. We also noted no significant hypotension. Acute, occasionally fatal hypotension has occurred following administration of HHT as a brief infusion at doses ≥ 6.4 mg/m² (L. Lev, personal communication). Our ability to administer cumulative doses of 18.75 mg/m² without serious toxic effects suggests that HHT can be more safely administered by continuous infusion. Because we excluded patients with cardiac arrhythmias or recent myocardial infarction, we have no information regarding the potential toxicity of HHT in this population.

On the basis of these data, we conclude that HHT can be administered safely by continuous infusion at 3.25 mg/m²/day, and we suggest this dose for phase II evaluation in patients with solid tumors. Routine measurements of pulse and blood pressure four to six times daily are recommended; however, more intensive cardiovascular monitoring is probably not warranted at this dose, given the absence of autonomic toxicity in this study. Acute toxic reactions, such as vomiting and hypotension, appear to be minimal using this dose and schedule.

Several studies have shown that HHT can produce complete remissions in patients with acute nonlymphoblastic leukemia (3-5). The antitumor activity of this drug in patients with other neoplastic diseases is unclear. Because the drug can be safely administered as a continuous infusion, we recommend a broad phase II evaluation of HHT in patients with leukemia and solid tumors.

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Phase II Evaluation of Amsacrine (*m*-AMSA) in Solid Tumors, Myeloma, and Lymphoma: A University of Arizona and Southwest Oncology Group Study[1]

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A phase II trial was conducted to determine the clinical activity of amsacrine (*m*-AMSA) in patients with heavily pretreated solid tumors, myeloma, and lymphoma at the University of Arizona Cancer Center. Additionally, *m*-AMSA was evaluated at other Southwest Oncology Group institutions in breast cancer, myeloma, melanoma, and oat cell cancer of the lung. At a dose of 120 mg/m² given iv every 28 days, 12 partial responses were observed in 221 patients evaluable for response. Some antitumor activity was observed in breast cancer (four responses of 65 patients), non-Hodgkin's lymphoma (three of nine), Hodgkin's disease (two of five), and sarcoma (two of 15). A partial response was also documented in one of two patients with cervical cancer. Among the 135 patients treated at the University of Arizona who were extensively evaluated for toxic effects, only myelosuppression and anemia were seen in a significant number of patients. At this dose and schedule, 29% of patients developed leukopenia of < 3000 cells/mm³, 16% developed a thrombocytopenia of < 100,000 cells/mm³, and 29% had an acute fall in hemoglobin of ≥ 2 g/100 ml. In addition, two patients suffered grand mal seizures which were not clearly drug-related. These results suggest that further study of *m*-AMSA in lymphoma, sarcoma, and cervical cancer is warranted. [Cancer Treat Rep 67:697–700, 1983]

Amsacrine (*m*-AMSA) is an acridine derivative which has demonstrated significant antitumor activity in several mouse tumors (1,2). Phase I studies conducted with *m*-AMSA have demonstrated a dose-limiting toxic effect of myelosuppression with bone marrow recovery usually occurring 18–24 days after drug administration (3,4). The maximum tolerated dose of *m*-AMSA was 90–120 mg/m² given either as a single dose or divided over 3 days. Phase II studies using *m*-AMSA in both hematologic malignancies and solid tumors have been ongoing. In this paper we report our results using *m*-AMSA in solid tumors, myeloma, and lymphoma.

PATIENTS AND METHODS

One hundred and thirty-five patients at the University of Arizona Cancer Center were entered on a phase II pilot study which was open to all patients with metastatic cancer, myeloma, or lymphoma. Subsequent to this activation, the Southwest Oncology Group (SWOG) began study #7963 to evaluate *m*-AMSA in breast cancer, myeloma, oat cell lung cancer, and melanoma. One hundred and twenty patients not treated at the University of Arizona were entered on this study and these results will also be reported here.

Eligibility criteria included relapse after standard chemotherapy, measurable disease, and a life expectancy of at least 6 weeks. The patients who were entered ranged from 17 to 84 years of age. Four patients had had no prior treatment with chemotherapy, including one patient with sarcoma, two with cholangiocarcinomas, and one with squamous cell carcinoma of the cervix. The previously untreated patient with sarcoma had

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significant heart disease precluding the use of any regimen containing doxorubicin. The other 130 patients treated at the University of Arizona had previously failed an average of 2.5 different chemotherapeutic regimens, including an average of five drugs/patient. All but 15 of the previously treated patients had received doxorubicin.

m-AMSA was administered at a dose of 120 mg/m² diluted in 500 ml of D_5W and infused iv over 1 hour every 28 days. Poor-risk patients (> 65 years of age), patients who had previously received abdominal or pelvic radiation therapy, or patients who had previously demonstrated significant myelosuppression with chemotherapy were treated with an initial dose of 90 mg/m². In addition, patients with abnormal liver function (a bilirubin > 3 mg/100 ml or an elevated alkaline phosphatase of SGOT above three times the normal rate) received an initial dose of 60 mg/m². Subsequent doses for all patients were based on a sliding scale according to toxicity.

Prior to the initiation of therapy, patients had a careful assessment of all measurable tumor as well as baseline blood cell counts and liver function testing. Patients were seen and the extent of their disease was evaluated every 4 weeks. An adequate trial of therapy with *m*-AMSA was considered to be two courses.

Criteria for response to therapy, except for myeloma, were as follows: *partial response*—a > 50% reduction of all measurable lesions lasting at least 2 months; *minor or mixed response*—a reduction in some lesions while other lesions remained the same or progressed; and *no response or disease progression*—gross tumor progression, the development of new lesions, or no tumor response after two courses of therapy. For patients with multiple myeloma, response criteria were those developed and used by the SWOG (5).

Patients who died < 3 weeks after the initiation of therapy were considered to be partially evaluable and were only evaluated for clinical characteristics and toxic effects. The duration of response to *m*-AMSA was calculated from the date of initiation of therapy until disease progression.

RESULTS

Of the 135 patients who were entered on this study at the University of Arizona, 101 (75%) were fully evaluable for response and toxic effects (table 1). Eighteen additional patients (13%) were evaluable for toxic effects but not for response. One hundred and seventy-four patients with myeloma, oat cell lung cancer, melanoma, and breast cancer were entered from other participating SWOG institutions, of which 120 (69%) were evaluable for response (table 1).

TABLE 1.—Tumor types treated with *m*-AMSA and response

Tumor type	No. of evaluable patients	No. of—		
		Partial responses (%)	Minor or mixed responses	No response or disease progression
Breast cancer	65	4 (6)	1	60
Oat cell lung cancer	39	0	0	39
Myeloma	34	0	0	34
Melanoma	28	0	3	25
Sarcoma	15	2 (13)	0	13
Non-Hodgkin's lymphoma	9	3 (33)	0	6
Non-oat cell cancer	6	0	0	6
Hodgkin's disease	5	2 (40)	0	3
Colon cancer	5	0	0	5
Bladder cancer	3	0	0	3
Cervical cancer	2	1 (50)	0	1
Ovarian cancer	2	0	0	2
Gastric cancer	2	0	0	2
Hepatoma	2	0	0	2
Cholangiocarcinoma	1	0	0	1
Pancreatic carcinoma	1	0	0	1
Thyroid carcinoma	1	0	0	1
Endometrial cancer	1	0	0	1
Totals	221	12 (5)	4	205

Among the 221 evaluable patients, 12 (5%) partial responses occurred. Four other patients exhibited mixed responses. The responses by tumor type are tabulated in table 1. In breast cancer, four partial responses (6%) were documented in the 65 evaluable patients. These responses lasted from 2 to 9 months. Three of the four partial responses occurred in patients with bone disease in whom bone pain abated, an elevated alkaline phosphatase normalized, and a bone scan demonstrated improvement (decreased activity). Serial bone radiographs in the metastatic areas were not performed. There was also one mixed response among the breast cancer cases.

Fifteen evaluable patients with sarcomas were treated with *m*-AMSA. These 15 patients included three with osteosarcoma, one with Ewing's sarcoma, and 11 with soft tissue sarcomas. There were two partial responses, both occurring in patients who had previously been treated with combination chemotherapy regimens containing doxorubicin. One patient with osteosarcoma had a > 50% decrease in pulmonary nodules after the first course of *m*-AMSA but refused further therapy, and a patient with spindle cell sarcoma had a partial response of enlarged lymph nodes and subcutaneous metastases lasting 7 months.

Fourteen patients with lymphoma (five with Hodgkin's disease, four with diffuse poorly differentiated lymphocytic lymphoma, two with diffuse histiocytic lymphoma, two with nodular poorly differentiated lymphocytic lymphoma, and one with systemic T-cell lymphoma) were treated with *m*-AMSA. In these disease categories, there were five partial responses, all occurring in patients with measurable lymph nodes. Two of the five patients with Hodgkin's disease experienced partial responses lasting 4 and 5 months. One of the two patients with nodular poorly differentiated lymphocytic lymphoma had a partial response lasting 2 months, and two of the four patients with diffuse poorly differentiated lymphocytic lymphoma had partial responses lasting 2 and 6 months. The two patients with diffuse histiocytic lymphoma and the one patient with systemic T-cell lymphoma failed to respond.

In other disease categories, one partial response was noted in a patient with cervical carcinoma, who had a > 50% reduction in the size of a pelvic mass lasting 3 months. No responses were seen in oat cell lung cancer (39 cases), myeloma (34), large cell cancer of the lung (five), colon cancer (five), bladder cancer (three), ovarian carcinoma (two), gastric cancer (two), hepatoma (two), squamous cell cancer of the lung (one), cholangiocarcinoma (one), pancreatic carcinoma (one), thyroid carcinoma (one), and endometrial carcinoma (one).

Toxicity

Extensive monitoring of toxic effects was carried out on the patients treated at the University of Arizona. The toxicity associated with *m*-AMSA therapy utilizing this dose and schedule was minimal except for myelosuppression and anemia. Of the 119 fully and partially evaluable patients at the University of Arizona, 29 (24%) experienced at least mild nausea with *m*-AMSA. Two of these patients experienced vomiting, and a third patient refused further therapy with *m*-AMSA secondary to nausea and vomiting during the first course. There were no skin rashes observed or documented cases of phlebitis secondary to the drug. Serum electrolytes and electrocardiograms were not monitored during therapy and no cardiovascular toxicity was documented. However, two patients experienced grand mal seizure activity. One patient with melanoma had an apparent seizure during the administration of her first course of *m*-AMSA. This patient subsequently was demonstrated to have brain metastases and died within 4 weeks. A second patient experienced a grand mal seizure at home after her second treatment with *m*-AMSA. The patient subsequently had a normal CT brain scan and received a third course of *m*-AMSA without additional problems.

Myelosuppression and anemia were the major toxic effects associated with *m*-AMSA. Leukopenia of < 3000 cells/mm³ of the total wbc count developed in 35 (29%) of the 119 fully and partially evaluable patients. Twenty patients (17%) developed wbc counts of < 1000/mm³. The lowest wbc count recorded was 300/mm³. There were three leukopenia-related deaths. All but one case of leukopenia occurred at doses of 90–120 mg/m². Thrombocytopenia of < 100,000 platelets/mm³ developed in 19 cases (16%). Fifteen patients (13%) had platelet counts of < 75,000/mm³ recorded, including seven (5%) with platelet counts < 25,000/mm³. The lowest platelet count recorded was 3000/mm³. Thrombocytopenia of < 100,000 platelets/mm³ was associated with all dose levels of drug. There were no significant bleeding episodes related to thrombocytopenia and no resultant deaths.

Thirty-four patients (29%) suffered a decrease in their hemoglobin levels by at least 2 g/100 ml while being treated with *m*-AMSA. In 19 patients (16%), this fall was at least 2 g/100 ml; in ten (8%), the fall was at least 4 g/100 ml; and in seven (6%), the fall was > 5 g/100 ml, with one patient experiencing a hemoglobin drop of 7.1 g/100 ml. In no case was there concurrent clinically detectable bleeding or other causes for an acutely falling hemoglobin level. The fall in hemoglobin occurred with the first or second course in all patients, and in 15 patients who received four or more courses of *m*-AMSA, the hemoglobin levels stabilized in 11 of the 15 by the fourth course. Twenty-six patients (22%) required transfusions. The cause of this acute fall in hemoglobin is unknown and no screening tests for hemolysis were performed.

DISCUSSION

In this phase II study, *m*-AMSA produced antitumor activity in extensively pretreated patients with breast cancer, sarcoma, and lymphoma (including Hodgkin's disease). Cabanillas et al (6) have also reported activity for *m*-AMSA in non-Hodgkin's lymphoma in adults, and Tan et al (7) have shown activity for *m*-AMSA in childhood lymphomas. The results from larger SWOG studies also suggest activity for *m*-AMSA in lymphomas (8), but no activity was found in advanced non-Hodgkin's lymphoma in a fourth study (9). Our finding of two partial responses in five patients with Hodgkin's disease treated with *m*-AMSA is the first published report of activity in this disease. *m*-AMSA merits further study in lymphoma.

In heavily pretreated patients with metastatic breast cancer, 6% of 65 patients achieved a partial response. This degree of activity is slightly less than that observed in two other breast cancer studies with *m*-AMSA (10,11), but is higher than the activity observed in a third report where no responses were documented (12). Overall, *m*-AMSA appears to be minimally active in breast cancer.

Although two of 15 patients with sarcomas achieved a partial response with *m*-AMSA, the activity of the drug in this disease category remains uncertain. At Memorial Hospital, 23 patients with advanced sarcomas were given *m*-AMSA and only one partial response was noted (13). At M. D. Anderson, 31 patients with sarcoma were given *m*-AMSA with one partial response and 13 stable disease responses noted (14). *m*-AMSA appears to have little activity in soft tissue sarcomas but additional study in bone sarcomas may be warranted.

Of the 12 partial responses documented, 11 were in patients who had previously been treated with doxorubicin. The 12th patient had cervical carcinoma and had not previously received any chemotherapy. Hence, there does not appear to be any significant degree of cross-resistance between doxorubicin and *m*-AMSA.

m-AMSA was a well-tolerated drug except for moderate myelosuppression. No clear cardiac toxicity was found in our study although two patients suffered apparent grand mal seizures. Careful monitoring of serum electrolytes and electrocardiograms may not be necessary when treating patients similar to those in this study. This monitoring may be required in more critically ill patients (15,16). A new finding was the development of significant anemia in 29% of our patients which necessitated transfusion in 22%. A suggestion of this toxic effect was noted in a phase II study with *m*-AMSA in lung cancer (17) but its prevalence and severity were not documented. This risk of *m*-AMSA therapy should be recognized and studied further in future studies to define its etiology.

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Phase I Study of Acivicin in Patients With Advanced Cancer[1,2]

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Acivicin is an antitumor antibiotic isolated from fermentation broth of *Streptomyces sviceus* (1). Acivicin is active against a variety of murine tumors and human tumor xenografts (1-3).

Structural similarities between L-glutamine and acivicin led to the observation that the agent is a potent inhibitor of L-glutamine utilization by L-asparagine synthetase, cytidine 5′-triphosphate synthetase, xanthine 5′-monophosphate aminase, and other L-glutamine-requiring enzymes (4). Clinical studies with L-glutamine antagonists revealed that these agents had limited but definite antitumor activity (5). DON (6) and azotomycin had remarkable activity against human tumor xenografts (5). On the basis of the preclinical activity and the clinical data on other L-glutamine antagonists, the initial clinical study of acivicin was undertaken.

MATERIALS AND METHODS

Acivicin was produced by the Upjohn Company, Kalamazoo, MI, and was supplied by the National Cancer Institute, Bethesda, MD. Ten-milliliter flint vials containing 25 mg of the lyophilized form of the compound, 25 mg of mannitol, and sodium hydroxide to adjust the pH to 6.0-8.0 were stored under refrigeration (2°C-8°C).

Patient entry in the clinical trial was based on our standard criteria (7). In the latter phase of the study, patients with pre-existing major-organ dysfunction and increased third-space fluid were entered at lower doses. Baseline studies were carried out in the usual manner (7).

The initial dose of 0.7 mg/m²/day × 5 corresponded to one-third the toxic dose low in dogs. The dose was administered as a rapid iv infusion over 10-15 minutes. Dose escalations were guided by modified Fibonacci search scheme. Toxic and therapeutic effects were evaluated according to Cancer and Leukemia Group B criteria.

RESULTS

Thirty-one patients, 17 men and 14 women, were included in the study; the median age was 58 years (range, 30-77). All patients had received prior chemotherapy and 16 had, in addition, received prior radiotherapy. The spectrum of disease categories included cancer of the head and neck (nine patients); gastrointestinal tract (six); breast (five); mesothelioma (three); kidney (two); ovary (two); and lung, adrenal gland, prostate, and fibrous histiocytoma (one each). The median performance status (Cancer and Leukemia Group B criteria) was 2 (range, 0-4).

Forty-five courses of therapy were given and were evaluable for immediate side effects. Because of early death (disease-related) and loss to follow-up, 41 courses in 28 patients were evaluable for subacute toxicity. Four patients received two courses, three received three courses, and one received four courses. Median interval between courses was 28 days (range, 21-35).

Myelosuppression and neurologic toxic effects were the major side effects (table 1). Myelosuppression was dose-related. At the 20-mg/m²/day dose level, all four patients who received the 5-day course developed leukopenia, with a wbc count $< 2 \times 10^9$/L. One patient developed epistaxis and petechiae, with a platelet count nadir of 1.7×10^{10}/L, requiring platelet transfusions. With these observations, we concluded that we had reached the maximally tolerated dose in our patient population. The median time to wbc count nadir was 12 days, and the median time to recovery was 19 days, ie, 7 days after reaching the nadir. The median time to the platelet count nadir was 14 days, and the median time to recovery was 21 days. Among seven patients with bone marrow abnormalities (tumor infiltration or hypocellularity), isolated cases of severe thrombocytopenia were noted. In patients

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TABLE 1.—Dose escalation of acivicin and toxic effects observed

Dose (mg/m²/day × 5)	No. of patients entered	No. of courses	No. of courses with nausea and vomiting/No. of evaluable courses	No. of courses with neurotoxicity	No. of evaluable courses for subacute toxicity	Median wbc count nadir (range), × 10^9 cells/L	Median platelet count nadir (range), × 10^9 cells/L	Hepatotoxicity
0.7	2	2	1 and 0/2	0	1	10.0	311	0
1.5	3	3	0 and 0/2	0	2	7.9 (3.8–11.9)	254 (127–380)	0
2.0	3	7	0 and 0/7	0	7	5.5 (3.4–9.8)	198 (160–268)	0
3.5	3	3	2 and 2/3	0	3	8.8 (4.1–8.9)	323 (208–435)	0
5.5*	4	4	2 and 2/4	2	3	7.3 (6.5–8.4)	144 (110–439)	0
9.0	6	10	4 and 2/7	6	10	5.4 (3.3–11.9)	202 (89–273)	2
12.0†	4	4	1 and 1/3	2	4	2.7 (1.7–5.0)†	255 (8–336)†	1
15.0‡	4	7	1 and 0/5	4	6	1.8 (1.5–6.1)	156 (6–200)‡	2
20.0§	5	5	1 and 1/3	3	5	1.6 (0.45–2.8)§	56 (17–340)	0

*1 patient only 1 dose because of neurotoxicity.
†1 patient received the drug on 4 days only and had a wbc count nadir of 5.0 × 10^9 cells/L and a platelet count nadir of 336 × 10^9 cells/L.
‡1 patient received the drug on 3 days only and had a wbc count nadir of 1.5 × 10^9 cells/L and a platelet count nadir of 192 × 10^9 cells/L.
§1 patient received the drug on 3 days only and had a wbc count nadir of 2.8 × 10^9 cells/L and a platelet count nadir of 72 × 10^9 cells/L.

who were treated with identical doses, the wbc and platelet count nadirs or the time to reach them did not differ between initial and subsequent courses.

Thirteen patients who received 17 courses at doses ≥ 5.5 mg/m²/day developed neurotoxicity. Severity or frequency did not increase with dose increments within the dose range studied. This was characterized by reversible asthenia (11 courses), disorientation (six), lethargy (three), depression (two), paranoia (two), amnesia (one), hostility (one), and transient unconsciousness (one). These symptoms developed on Days 1–4, most commonly on Day 3, and cleared spontaneously within 8 hrs to 6 days. No permanent neurologic dysfunction was noted. Neurotoxicity was more common in patients requiring opiates for analgesia (all of 17 evaluable courses with neurotoxicity vs five of 12 courses with neurotoxicity) and in those with increased third-space fluid (six of 17 courses vs one of 12). Performance status, serum albumin levels, hepatic dysfunction, or degree of myelosuppression had no correlation. Abnormal EEG recordings showing diffuse slowing consistent with metabolic encephalopathy were noted in two patients.

Mild and reversible hepatotoxicity was noted following five courses in three patients and recurred in two patients when re-treated. The presence of mild hepatic dysfunction (four patients) did not enhance myelosuppression or neurotoxicity. Mild transient diarrhea occurred in two patients.

No partial or complete responses were noted. One heavily pretreated patient with carcinoma of the ovary had improvement lasting 7 weeks at a dose of 15 mg/m²/day × 5. Two patients with breast cancer and one with prostatic cancer had a decrease in bone pain caused by metastatic disease. A patient with pancreatic carcinoma with liver metastases had a mixed response with a decrease in the pancreatic mass and increase in hepatic masses.

DISCUSSION

The present study establishes that acivicin given as an iv infusion over 10–25 minutes at a dose of 20 mg/m²/day × 5 is the maximally tolerated dose in man and that the dose-limiting toxic effect is myelosuppression. The doses of 12–15 mg/m²/day × 5 were well-tolerated by patients with no major-organ dysfunction. Patients with compromised bone marrow reserve should be treated with an initial dose of 9.0 mg/m²/day × 5. While patients with compromised liver function were reported to have slow drug clearance (8), mild to moderate pre-existing hepatic dysfunction did not seem to be a major risk factor.

The exact nature of the acivicin-induced neurotoxicity is unknown. It is possible that acivicin, by blocking L-glutaminase activity in the central nervous system (4), lowers the production of L-glutamate, an important factor in a number of metabolic processes in the brain (9) and excitatory neurotransmitters (10). It is of interest to note that morphine reduces excitation by L-glutamate on putative transmitters of spinal cord sensory neurons (11). Alternatively, acivicin or its metabolites may themselves be toxic to the central nervous system as are ibotenic acid (12) and muscimol (12,13), structural analogs of acivicin. Our observation in a patient with pancreatic cancer is of interest in light of the fact that the rodent pancreas is known to trap and utilize L-glutamine at a rate greater than virtually any other organ (4). *Acinetobacter* glutaminase-asparaginase enhanced the effects of acivicin (14). These observations indicate that further study of this compound alone in selected neoplasms or as a biochemical modifier in combination with other L-glutamine inhibitors would be of value.

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High-Dose Chlorozotocin in Lung Cancer: A Southwest Oncology Group Phase II Study[1,2]

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Chlorozotocin was administered as an iv bolus at a dose of 200–225 mg/m² every 6 weeks for good-risk patients (100 mg/m² for poor-risk patients) with lung cancer. Only three of 71 fully evaluable patients responded; 12 had stable disease. Myelosuppression was acceptable and no other dose-limiting toxic effects were reported. Chlorozotocin is inactive in non-small cell lung cancer. [Cancer Treat Rep 67:705–707, 1983]

Chlorozotocin (CZT) is a chloroethyl derivative of streptozocin, with less myelotoxicity than other nitrosoureas in preclinical systems (1). Phase I studies (2,3), however, revealed dose-limiting myelotoxicity. The Southwest Oncology Group completed accrual for this phase II study in lung cancer in 1979.

MATERIALS AND METHODS

Patients with histologically proven advanced lung cancer and measurable lesions were eligible if they had an expected survival of 6 weeks, a wbc count $\geq 4000/\mu l$, a platelet count $\geq 100{,}000/\mu l$, and serum creatinine and bilirubin values ≤ 3.0 mg/dl. No minimum performance status (PS) was included in the eligibility criteria. No prior treatment within 3 weeks was allowed. Signed informed consent was obtained. Medical history and physical examination, appropriate x-rays, recording of weight and PS, cbc, platelet count, fasting chemistry panel, and 2-hour postprandial glucose were required within 7 days of starting therapy. Weekly cbc and platelet counts were requested. All pretreatment tests were repeated every 6 weeks.

Patients with no prior chemotherapy and normal hepatic and renal functions were to receive CZT at a dose of 225 mg/m², while those with good tolerance of prior therapy (no severe myelotoxicity or treatment delays), no prior nitrosourea and/or mitomycin, and normal hepatic and renal functions received 200 mg/m². Other patients received 100 mg/m². Treatment was repeated at 6 weeks in patients with stable or responding disease if they had recovered from toxicity. A dose escalation of 10% was prescribed if no myelosuppression occurred with the previous dose. A dose reduction of 25% was used when wbc and platelet count nadirs were 1000–2000 and 25,000–49,000/μl, respectively. A dose reduction of 50% was used for patients with more severe myelosuppression.

A partial response (PR) was defined as a $\geq 50\%$ reduction in the product of perpendicularly measured diameters of all measurable lesions for ≥ 30 days. Disease improvement was defined as tumor reduction less than a PR. Stable disease was defined as lack of tumor progression for ≥ 6 weeks. Tumor progression was defined as a 25% increase in tumor area. Treatment was continued until disease progression unless serious toxicity or patients' wishes intervened.

RESULTS

Eighty-three patients were registered. No data were received for two patients and two were never treated;

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79 were fully or partially evaluable. Of these 79 patients there were 34 with squamous cell carcinoma, 25 with adenocarcinoma, 16 with large cell anaplastic carcinoma, and four with small cell anaplastic carcinoma. Eight partially evaluable patients died within 16 days of treatment, leaving 71 fully evaluable patients. Of these 71 patients, 13 had received no prior therapy, 22 had had prior radiation therapy only, and the remainder had had prior chemotherapy with or without radiation therapy. Fourteen patients with radiation therapy but no prior chemotherapy were entered at a dose of 200 mg/m², a minor protocol deviation. Prior chemotherapy was highly diverse, but often included doxorubicin alone or in combination with one or more other drugs. Eighty-one percent of patients were males; 13% were nonwhite.

Response Data

PRs were reported in one patient each with adenocarcinoma, squamous cell carcinoma, and large cell anaplastic carcinoma, with durations of 100, 75, and 139 days, respectively. All responders received 200 mg/m² of CZT as shown in table 1. One of the 35 patients without prior chemotherapy and two of 44 patients with previous chemotherapy achieved PR. The PR rate was 4% and the improvement or stable disease rate was 15%. Table 2 shows the effect of PS on the likelihood of benefit with CZT and on median and maximum survival. Even asymptomatic patients were unlikely to respond or to live > 4 months, and no PS group was free of "early" deaths.

Toxicity Data

Myelosuppression was related to dose of CZT and to prior treatment. Table 3 showed no trend toward cumulative toxicity if the described dose adjustment guidelines were used. The 200-mg/m² dose level for the patients with radiation therapy and/or well-tolerated prior chemotherapy produced severe to life-threatening leukopenia and/or thrombocytopenia in 16% and 31% of the first courses, respectively (including the one toxic death of this series). The 225- and 100-mg/m² dose levels were free of severe myelotoxicity through the first two courses of treatment.

Five patients had mild proteinuria after one to two courses of CZT and one patient developed mild creatinine elevation (1.6 mg/dl) after five courses concomitant with tumor progression. Five patients from a single institution developed mild to moderate diarrhea; no other institution reported additional cases. One case each of mild skin rash and possible drug-related cholestasis was reported. Severe vomiting was reported in only two patients (2.5%). No islet-cell dysfunction occurred.

DISCUSSION

Only three of 71 fully evaluable patients with non-small cell lung cancer responded in our series, confirming other groups' results with CZT in these tumors (4,5). No subset of patients likely to benefit with this treatment could be identified after analysis by cell types, PS, presence or absence of prior radiation

TABLE 1.—CZT dose vs response of lung cancer by cell type

Cell type	Dose (mg/m²)	No. of patients with— PR	Stable disease or improvement	Progressive disease	Early death
Squamous cell carcinoma	225	—	3	5	1
	200	1	3	11	3
	100	—	—	7	—
Adenocarcinoma	225	—	3	5	1
	200	1	1	12	—
	100	—	—	2	—
Large cell anaplastic carcinoma	225	—	2	1	—
	200	1	—	5	1
	100	—	—	5	1
Small cell anaplastic carcinoma	225	—	—	—	—
	200	—	—	2	1
	100	—	—	1	—
Totals		3	12	56	8

TABLE 2.—Karnofsky PS in lung cancer patients treated with CZT: effects on survival and response

Karnofsky PS	No. of patients*	Median survival in days (range)	% of patients responding or with stable disease
90-100	7	144 (15-323+)	43
70-80	22	125 (3-281)	18
50-60	24	76 (8-183)	17
30-40	21	34 (2-138)	5
10-20	3	55 (16-106)	—

*Excludes 2 patients with unrecorded PS.

TABLE 3.—Dose and course vs myelosuppression

Dose (mg/m^2)	Course No.	No. of evaluable patients	Wbc count nadir (cells × $10^3/\mu l$)		Platelet count nadir (cells × $10^3/\mu l$)	
			Median	Range	Median	Range
225	1	16	5.7	2.4-13.13	175	60-670
	2	8	4.9	2.6-7.8	203	67-425
	3	6	4.4	3.2-5.5	170	84-268
	4	2	—	1.8-6.8	—	24-191
200	1	31	4.0	0.5-12.7	88	15-486
	2	11	5.7	2.8-8.7	88	36-524
	3	2	—	3.7-7.9	—	145-147
100	1	14	6.8	3.3-9.8	250	81-517
	2	6	7.7	4.3-11.5	279	163-452

therapy and/or chemotherapy, or combinations of these factors. We conclude that CZT is inactive in these cancers.

Since our series included only four patients with small cell lung cancer, the effect of CZT in this cell type remains undetermined.

Myelosuppression was most pronounced in previously treated patients who received 200 mg/m^2 of CZT. As noted by others (2,3), thrombocytopenia occurred more frequently than did leukopenia. Mild nephrotoxicity was noted in six patients, but was not clinically important. The dose schedules used are safe and applicable to further studies in other malignancies.

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Conservative Management Without Alkylating Agents of Squamous Cell Anal Cancer Using Cyclical 5-FU Alone and X-Ray Therapy[1]

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We have treated 11 patients with squamous cell anal carcinoma using a regimen of repeated cycles of 120-hour infused 5-FU (25 mg/kg/24 hours) and x-rays. Total radiation doses were between 3000 (palliative) and 4750 (curative) rads. Unlike all other equivalent combined-modality studies, no alkylating agent or drug other than 5-FU was used. With follow-up between 1 and 4 years, there has been only one local recurrence (coincident with terminal disseminated disease in a stage III patient treated palliatively). All other patients have maintained local control. Inclusion of alkylating agents (eg, mitomycin) in this approach can be questioned if the patient is willing to accept somewhat greater acute but reversible toxic effects. [Cancer Treat Rep 67:709–712, 1983]

Several recent reports have indicated that combined-modality therapy may be considered a therapeutic equivalent to radical surgery in anal cancers (1–3). To date, all of these trials have used some combination of infused 5-FU and bolus mitomycin together with external-beam radiation. A subcurative total x-ray dose of 3000 rads has usually been used. In the trial reported here, we have used a similar regimen but have omitted the mitomycin. The results appear as good as other studies for early disease (stages I, II, and IIIA) and can be considered as another option by oncologists wishing to avoid the carcinogenic potential of alkylating drugs.

MATERIALS AND METHODS

Patient characteristics, toxic effects, and current results are shown in table 1. All patients had invasive squamous cell cancer of the anus, staged according to the scheme of Singh et al (4) using adequate examination and surgical biopsy under anesthesia with lymph node biopsy if indicated.

Our regimen has been a combination of 120-hour infused 5-FU and external-beam x-rays administered in a *cyclical* fashion. The chemotherapy component has been 5-FU administered in the hospital through an IVAC or equivalent pump at 25 mg/kg/24 hours for a total of 120 hours/treatment cycle. The radiation therapy component of *each cycle* consisted of four daily treatments of 250 rads each, ie, 1000 rads/cycle. All radiation treatments included the entire pelvis and perineum, with an effort to assure that the inguinal lymph nodes were treated bilaterally in their entirety. All treatments were given on a 6-mev linear accelerator with the tumor dose calculated at mid-plane. Neither bolus nor perineal boosts were utilized.

All infusion cycles were started on Monday, as was the first radiation treatment of each cycle. The *minimum* rest period between cycles was 9 days; this period was routinely extended until all toxic effects had subsided. The 5-FU/x-ray schedule was used in accordance with our approach of 5-FU as a radiosensitizer, which postulates that radiosensitization (5) by the drug is a postradiation phenomenon requiring at least 24 hours after each x-ray exposure to fully develop (6). The number of treatment cycles and the total radiation dose varied according to the intent of therapy in each case. Some patients treated early in the study received less than adequate minimal radiation doses for cure (at least 4000 rads); nevertheless, they became unsuitable or unwilling to accept subsequent radical surgery because of complete clearance of disease. Total radiation doses for each patient are given in table 1 and may be compared to each patient's stage.

Four weeks after the last cycle, all patients underwent wide excisional biopsy and had a groin dissection if there was suspected inguinal lymph node involve-

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TABLE 1.—Patient characteristics, response, and status

Patient No.	Disease stage	Cycles (rads)/days	Local response, clinical/surgical*	Regional response, clinical/surgical*	Toxic effects†				Current status‡
					Mucosal	Hematologic	Skin	GI	
1	I	4 (4000)/67	CR/CR	—	2+	0	3+	1+ UGI, 2+ LGI	NED: 2 yrs, 7 mos
2	I	4 (4000)/76	CR/CR	—	0	0	2+	2+ LGI	NED: 2 yrs, 8 mos
3	I	5 (4250)/72	CR/CR	—	2+	1+	2+	1+ UGI, 2+ LGI	NED: 2 yrs, 9 mos
4	IIB	4 (4000)/60	CR/CR	—	1+	0	2+	2+ UGI	NED: 2 yrs, 7 mos
5	IIB	4 (4000)/74	CR/CR	—	3+	0	2+	2+ UGI, 2+ LGI	NED: 2 yrs, 8 mos
6	IIB	4 (4000)/60	CR/CR	—	0	0	3+	1+ LGI	NED: 2 yrs, 11 mos
7	IIB	3 (3000)/46	CR/CR	—	0	0	3+	1+ UGI (scrotal edema)	NED: 4 yrs
8	IIIB	4 (4000)/67	CR/CR	CR/CR	1+	0	2+	2+ UGI, 2+ LGI	NED: 2 yrs
9	IIIB	3 (3000)/31	CR/PR	—	0	0	2+	2+ UGI, 2+ LGI	Died, 7 mos with lung, brain metastases, local recurrence
10	IIIB	3 (3000)/32	CR/CR	CR/CR	1+	0	3+	1+ LGI	Died, 2 yrs, 4 mos; NED in pelvis, local
11	IVB	5 (4750)/67	CR/ND	CR/ND	0	0	3+	2+ UGI	Died, 8 mos systemic carcinoma; ? groin recurrence; local NED

*CR = complete response, PR = partial response, and ND = not done.

†Toxicity criteria—mucosal: 1+ = minimal, 2+ = soft foods only, 3+ = liquids only; hematologic: 1+ = neutrophil count, 1000–1699/mm³; skin: 1+ = erythema, 2+ = dry desquamation, 3+ = moist desquamation; upper and lower gastrointestinal (UGI,LGI): 1+ = nausea or diarrhea well-controlled by medications, 2+ = poorly controlled.

‡NED = no evidence of disease.

ment (stage IIIB) prior to treatment. One patient (stage IV with pretreatment liver metastases) treated palliatively, who obtained a complete local-regional response, did not undergo any surgery.

RESULTS AND DISCUSSION

The results of therapy are also shown in table 1. All patients reached a complete local clinical response which was confirmed in all but one case by wide (excisional) biopsies. None of the patients who had clinical evidence of regional disease (inguinal node involvement) had any tumor in the node resection specimen. Thus, all but one patient achieved a surgically confirmed complete local-regional response. With a follow-up period of between 2 and 4 years, only one patient has shown a subsequent local (anal) recurrence. This occurred immediately before death of the stage IIIB (initially) patient (No. 9), treated palliatively (3000 rads in three infusion cycles), who had residual tumor in the posttreatment biopsy. Two other patients presenting with advanced disease have also died of systemic cancer without local recurrence.

Toxic effects of patients in the program are shown in table 1. The primary toxic effect was dermal, with six of the 11 patients developing patchy areas of moist desquamation during the course of treatment, which always completely healed following cessation of therapy. Intestinal toxic effects were less severe and consisted largely of nausea with occasional vomiting. (In our toxicity grading scheme, patients whose nausea is not *completely* controlled by antiemetics are classed as grade II toxic.) A few patients had diarrhea of an intermittent nature. Approximately one-half of the patients had some form of *systemic* 5-FU infusional toxicity, largely stomatitis. There were no significant hematologic toxic effects despite whole-pelvis radiotherapy combined with significant amounts of 5-FU.

These results may be compared to the results of others in which polychemotherapy, including an alkylating agent, has been used in combination, usually with lower doses of radiation (1–3,7–10). The results of both approaches are, in our opinion, equivalent thus far in terms of response rate. The responses we have observed appear durable, but longer follow-up is obviously desirable. This is true for most existing studies; only the study of Nigro et al (2) gives any long-term data for conservatively treated patients (ie, only one patient has been reported disease-free for 5 years thus far). Other studies which report good response rates for similar therapy do not have significant patient numbers in the group treated with conservative surgery (7–10); therefore, these studies are not comparable when the basic consideration is whether or not radical surgery and a permanent colostomy are at issue.

Our regimen appears more acutely toxic when compared to lower (x-ray) dose, mitomycin-containing regimens, particularly with respect to acute dermal toxic effects and, perhaps, upper gastrointestinal toxic effects. In this respect, our study and data resemble most closely those of Cummings et al (3) who also used full-dose radiation with this level of 5-FU. Cummings et al included mitomycin and employed 5-FU during only a part of the x-ray program. Both dermal and intestinal toxic effects were noteworthy in their study, as in the one reported here.

We feel the omission of an alkylating agent component may be worthwhile, since this should significantly reduce the potential for late treatment-induced malignancies. To date, there have been no significant late effects noted in the bowel, and subcutaneous skin changes appear essentially the same as those seen with radiation alone. However, a larger number of patients and more prolonged follow-up will be required to accurately determine the precise level of secondary problems.

These results have been obtained with higher doses of radiation than those used in most other studies and with a 5-FU infusion load now known to be in the region where clearance is nonlinear in many patients (10–12). Serial determinations of serum 5-FU may permit therapeutic levels of the drug to be used with more manageable toxicity. Until further investigations have been completed, the use of this particular regimen can be considered by oncologists who feel that a somewhat higher incidence of acute, reversible side effects may be more acceptable than exposure of the patients to agents known to have significant carcinogenic potential (13–15).

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Phase II Evaluation of Mitomycin and Cisplatin in Advanced Esophageal Carcinoma[1,2]

Paul F. Engstrom,[2,*] Philip T. Lavin,[2] and David J. Klaassen[2,3]

In October 1978 the Eastern Cooperative Oncology Group (ECOG) initiated a phase II protocol (EST: 2278) designed to screen agents for activity in patients with squamous cell carcinoma of the esophagus (SCE). Mitomycin was selected because Cook and Bradner (1) reported a 15% subjective response rate in esophageal carcinoma. Cisplatin was studied because of its activity against squamous cell carcinomas in the head and neck region (2).

MATERIALS AND METHODS

All patients had measurable histologically confirmed advanced or recurrent SCE. In previously irradiated patients, the marker lesion was located outside of the therapy portals. The initial patient performance status was ≤ 3 (as defined by ECOG criteria). Either intestinal or iv hyperalimentation was used if the caloric intake was < 1500 calories/day during the treatment program. All patients met the following pretreatment laboratory criteria: wbc count > 4000/mm³, platelet count > 100,000/mm³, BUN < 25 mg/dl, creatinine < 1.5 mg/dl or creatinine clearance < 75 ml/min, bilirubin < 1.5 mg/100 ml, and SGOT < 60 IU/ml. All patients signed an institutional consent form prior to study and were stratified by performance status (0–1 or 2–3), prior chemotherapy (yes-no), or prior radiotherapy (yes-no). Patients were randomized to receive either mitomycin or cisplatin.

Based on the report of Godfrey and Wilbur (3), mitomycin was administered at a dose of 20 mg/m² iv every 4 weeks × 2, then every 6 weeks until evidence of disease progression or serious toxicity. Fifty percent of the calculated dose was administered on subsequent scheduled visits if the platelet counts were between 75,000 and 100,000/mm³ or the wbc counts were between 2000 and 4000/mm³. No chemotherapy was given for a wbc count < 2000/mm³ or a platelet count < 75,000/mm³. Cisplatin was administered iv every 3 weeks at a dose of 50 mg/m², with 2 L of iv 5% dextrose and 0.45% NaCl, 40 mg of furosemide, and 12.5 g of mannitol to ensure adequate diuresis. Antinauseant agents were given at the discretion of the investigator. The calculated dose of cisplatin was modified to 50% for a wbc count nadir < 2000/mm³ and a platelet count nadir < 75,000/mm³, and no cisplatin was given for a BUN or creatinine > 1.5 × baseline or a creatinine clearance > 75 ml/min. The investigator could discontinue cisplatin for neurotoxicity including seizures, peripheral neuropathy, or ototoxicity. Audiograms were performed before treatment and were repeated every 3 months during cisplatin therapy.

RESULTS

Standard ECOG criteria for toxicity and solid tumor response were used in patient evaluation. Fifty-four of the 59 patients were evaluable. Chemotherapy toxicity

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was assessed for 53 patients. Forty-eight patients who had not received chemotherapy prior to study entry were evaluated for response. Study results and patient characteristics are summarized in table 1.

Antitumor activity was demonstrated for both treatment programs. The mitomycin treatment program had a 42% (ten of 24 patients) partial response rate, a response duration range of 4-23 weeks (median, 12), and a survival duration of 2-126 weeks (median, 14). The cisplatin treatment had a 25% (six of 24 patients) partial response rate, a response duration range of 3-49 weeks (median, 16), and a survival duration of 3-71 weeks (median, 18).

Five patients treated with mitomycin developed ECOG grade 4 complications: two with life-threatening lung infections, two with life-threatening neutropenia, and one with life-threatening thrombocytopenia (a platelet count nadir < 25,000/mm³). Grade 3 toxicity was the worst degree for 14 additional patients; this included eight instances of hematologic toxicity with mitomycin and two instances of severe nausea and vomiting associated with cisplatin. No patient had the cisplatin discontinued because of renal toxicity or severe incapacitating neurotoxicity.

DISCUSSION

This study confirmed that mitomycin has antitumor activity in SCE. However, the usefulness of this agent was compromised by the dose-dependent hematologic toxicity, particularly cumulative thrombocytopenia, anemia, and leukopenia. The short median survival duration in mitomycin-treated patients could also be due to the relatively large proportion of patients with liver metastases. Further studies in SCE using mitomycin in a less toxic dose are indicated. Mitomycin (10 mg/m² on Day 1) has been successfully combined with 5-FU (1000 mg/m²/24 hours on Days 1-4 and 29-32) and irradiation (5000-6000 rads) in previously untreated patients with SCE (4).

Cisplatin at doses of 50 mg/m² every 3 weeks is an active drug for advanced SCE. Moderate nausea and

TABLE 1.—Patient characteristics and treatment results

	Treatment	
	Mitomycin	Cisplatin
No. of patients—		
Entered	32	27
Evaluated for toxicity	28	25
Evaluated for response*	24	24
Evaluated for survival*	26	24
Cycles completed		
1	17%	8%
2	38%	40%
3	24%	8%
4	21%	40%
Median age (range)	59 yrs (44-75)	58 yrs (46-74)
Females	24%	16%
Prior therapy		
Radiation therapy	52%	64%
Chemotherapy	10%	4%
Performance status		
Ambulatory (ECOG 0 to 1)	72%	64%
Liver metastases (positive)	34%	12%
Lung metastases (positive)	38%	32%
Toxicity		
Severe vomiting, emesis	7%	8%
Grade 4 neutropenia (< 1000 cells/mm³)	8%	0%
Grade 4 thrombocytopenia (< 25,000 cells/mm³)	4%	0%
Response*		
> 50% tumor response	42%	25%
Median response duration	12 wks	16 wks
Survival*		
Median survival duration	14 wks	18 wks

*Patients who had received no prior chemotherapy.

vomiting but no significant neurologic or hematologic toxicity was seen with this schedule. There are three additional reports on the efficacy of cisplatin as a single agent in SCE: Ravry et al (5) achieved a 40% (four of ten patients) partial response rate with 90 mg/m² every 21 days; Panettiere et al (6) noted two complete responses and four partial responses in 19 evaluable patients using 50 mg/m² on Days 1 and 8 every 28 days; and Davis et al (7) obtained only one response lasting 4 weeks in 17 patients using 3 mg/kg (110 mg/m²) iv every 4 weeks. Cisplatin has been incorporated into two effective three-drug regimens for SCE: cisplatin, vindesine, and bleomycin (29 of 53 patients, 55% response rate) (8); and methotrexate, bleomycin, and cisplatin (five of ten patients, 50% response rate) (9).

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Progesterone Receptor Level as a Predictor of Response to Megestrol Acetate in Advanced Breast Cancer: A Retrospective Study[1]

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Megestrol acetate (160 mg/day) produced a response rate of 44% in a retrospective series of 39 evaluable patients with advanced breast cancer. The estrogen-receptor (ER) level was > 10 fmols/mg of protein in 28 patients, and the progesterone-receptor (PR) level was > 10 fmols/mg of protein in 26 patients. ER and PR levels, age, and disease-free interval were analyzed for their relationship to response. The PR was the single best predictor of response to megestrol acetate; the addition of ER added 2% to the predictive accuracy rate of PR alone. [Cancer Treat Rep 67:717–720, 1983]

Megestrol acetate, a semisynthetic 11-hydroxyprogesterone derivative, is a progestational agent which is active in advanced breast cancer (1–4). Determination of the estrogen receptor (ER) has been shown to have value in predicting response to hormonal therapy in breast cancer (5). More recent reports indicate that determination of the progesterone receptor (PR) plus the ER increases the ability to predict response to endocrine manipulations (6–8).

The response rate to megestrol acetate in advanced breast cancer has been correlated with ER and PR determinations in only one series of patients; no correlation was found (9). We report the results of a retrospective analysis in which receptor levels and other potential predictive variables were correlated with response in a series of 39 evaluable patients with breast cancer treated with megestrol acetate.

MATERIALS AND METHODS

Patients.—The records of 43 consecutive patients with breast cancer who had been treated with megestrol acetate (160 mg daily) and who had known ER and PR levels and measurable or evaluable disease were examined retrospectively. All such patients treated at our institution were included, regardless of performance status, except for four who were considered inevaluable: one patient was eliminated from the series because of inadequate tumor measurements, one was eliminated because the PR was not measured until after the trial of megestrol acetate, and two died within 10 days of starting therapy. The final group of patients, which included 34 with measurable and five with evaluable disease, consisted of 37 postmenopausal women and two men. Twelve patients had received previous additive hormonal therapy and five had responded.

Definition of response.—Eastern Cooperative Oncology Group response criteria were used to determine the therapy results. Duration of response was dated from the start of therapy to the time of disease progression (10). A response was classified as stable, partial, or complete only if its duration was ≥ 12 weeks.

Receptor measurements.—Approximately three-quarters of the ER and PR levels were determined in our institution by a dextran-coated charcoal assay as reported previously (11). PR was determined with R 5020, in the presence of 20 mM sodium molybdate (12). The rest of the ER and PR values were from outside laboratories, primarily Mason-Barron, and were also determined by the dextran-coated charcoal assay.

RESULTS

Response

Among the 39 evaluable patients, there were five complete responses and 12 partial responses. The overall objective response rate was 44% (41% if early deaths are included). An additional nine patients (23%) had stable disease. Response by site of disease was as follows: soft

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tissue, 16 of 29 patients (55%); pulmonary, four of ten (40%); and osseous, two of five (40%). There were no patients with liver metastases in this series.

Median time to response was 12 weeks; median duration of response was 69 weeks for complete responders and 31 weeks for partial responders. The objective response to megestrol acetate (ER or PR) for the various receptor categories was as follows: 14 of 23 patients who were ER-positive and PR-positive; one of five who were ER-positive and PR-negative; one of three who were ER-negative and PR-positive; and one of eight who were ER-negative and PR-negative. Receptor levels > 10 fmols/mg of protein were considered positive.

ER and PR levels, age, and disease-free interval for responders and nonresponders are shown in figures 1 and 2, which indicate an apparent difference in ER and PR levels for responders compared to nonresponders. These relationships were examined further by applying a two-group linear discriminant analysis (13) using BMDP7M of the Biomedical Computer Program series P (14). The only variable retained by this analysis was the PR level (Cannonical r = 0.58).

When examined separately, both the ER (r = 0.49; $P < 0.001$) and the PR (r = 0.58; $P < 0.0001$) were related to response category. Since regression and analysis of variance are formally the same (13), this indicates that the groups differ on both measures, as suggested by figure 1. The greater r value suggests that the PR is more strongly related to response category. ER and PR levels were strongly related to each other (r = 0.79: $P < 0.0001$). However, adding the ER value to the prediction equation (13) increased the prediction accuracy rate by 2%. This is consistent with the fact that only the PR was retained in the linear discriminant analysis described in the previous paragraph (13,14).

Patient age and disease-free interval (fig 2) were unrelated to response (r = 0.17 and r = 0.08, respectively) and added nothing to the prediction equation.

Toxicity

The most common side effect was weight gain (> 10% of body weight in six patients). Two patients experienced withdrawal bleeding and one had minimal vaginal bleeding during megestrol acetate administration. Two patients developed mild pedal edema, and one had an exacerbation of hypertension requiring adjustment of antihypertensive medications. No patient discontinued megestrol acetate because of drug toxicity.

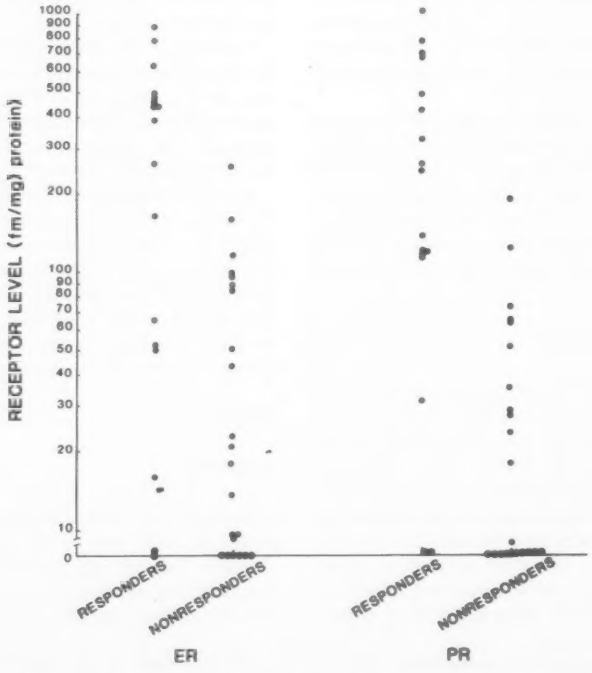

FIGURE 1.—ER and PR levels (fmols/mg of protein) for responders and nonresponders.

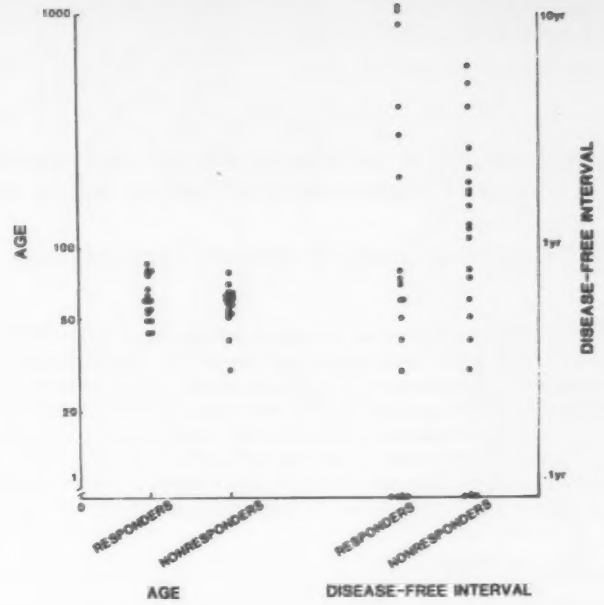

FIGURE 2.—Age and disease-free interval for responders and nonresponders.

DISCUSSION

The overall objective response rate of 44% to megestrol acetate is higher than previously reported (1–4), and this probably reflects the high proportion of receptor-rich tumors in our patients. Unlike Tuelings et al (9), we have shown a quantitative relationship between ER and PR values and response to megestrol acetate. The discrepancy between our results and those of Tuelings et al (9) may be attributable to their use of low-temperature agargel electrophoresis rather than the dextran-coated charcoal assay.

Horwitz et al (15) originally proposed the hypothesis that determination of the PR would improve the ability to predict response to hormonal therapy. Since that time several investigators (6–8) have reported that the likelihood of response to hormonal therapy is highest for patients whose tumors contain both ER and PR. Our observation that the quantitative PR level was the single best predictor of response to megestrol acetate lends additional support to this concept. More data, particularly from prospective studies, are needed to define the relationship between response to specific hormonal therapies and the quantities of ER and PR.

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MVP (Mitomycin, Vinblastine, and Progesterone): A Second-Line Regimen in Ovarian Cancer With a High Incidence of Pulmonary Toxicity[1]

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Thirteen patients with epithelial ovarian cancer refractory to primary combination chemotherapy regimens were treated with a three-drug combination called MVP: mitomycin, vinblastine, and progesterone. None of the patients had an objective response to therapy. Five of the 13 patients (39%) developed signs and symptoms consistent with drug-induced pulmonary toxicity following therapy with MVP and histologic confirmation was obtained in two patients. Mitomycin and vinblastine therapy in heavily treated patients with ovarian cancer may be associated with a higher risk of pulmonary toxicity than that previously reported for patients with other tumors. [Cancer Treat Rep 67:721–722, 1983]

The primary treatment of advanced ovarian cancer with combination chemotherapy regimens has resulted in overall response rates of 60%–80% (1,2). In contrast, the response rate to second-line therapy is only 6%–10% and the median survival for patients who relapse after induction chemotherapy is only 4–6 months. We instituted a trial of mitomycin, vinblastine, and progesterone (MVP) in patients with refractory ovarian cancer based on (*a*) recent reports that mitomycin was an active agent in ovarian cancer (3); (*b*) the demonstration that vinblastine in vitro had activity against human ovarian cancer cells; (*c*) the combination of mitomycin and vinblastine was demonstrated to have a 40% response rate in heavily treated patients with breast cancer (4); and (*d*) the observation that progestational agents appear to have activity in some patients with refractory ovarian cancer (5).

METHODS

All of the patients had received a primary combination chemotherapy regimen [either CHEX-UP (5) or HexaCAF (1)],[4] and seven of the patients had received a variety of second-line regimens prior to treatment with MVP. Most of the patients had a partial response to the prior regimen. None of the patients had clinical evidence of lung disease at the initiation of MVP. Mitomycin (20 mg/m^2) was given iv on Day 1 and vinblastine (0.15 mg/kg) was given iv on Days 1 and 21 with medroxyprogesterone acetate (200 mg orally, twice a day) administered continuously. Mitomycin and vinblastine were repeated every 6–7 weeks depending on hematologic recovery.

RESULTS

Twenty-nine cycles of MVP were administered to 13 patients with refractory ovarian cancer, with no objective responses. All of the patients experienced nausea and vomiting during therapy. Five of the patients developed platelet counts < 50,000/μl, and two had wbc counts < 1.5/μl.

Pulmonary Toxicity

Five of 13 patients (39%) developed respiratory distress while receiving therapy with MVP after mitomycin doses ranging from 41 to 93 mg (table 1). Chest x-ray findings demonstrated bilateral diffuse interstitial infiltrates. Three of the patients (one of whom received steroids) had a gradual improvement after cessation of mitomycin. One patient developed progressive respiratory failure. She initially had a response to steroid therapy but developed a fatal superimposed necrotizing bacterial pneumonia. One patient had an acute respiratory death. She had malignant pleural effusions prior to mitomycin therapy and her terminal respiratory failure clinically and radiologically was compatible with pulmonary

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[4]*See* table 1 for definitions.

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TABLE 1.—Pulmonary toxicity of patients with ovarian cancer treated with MVP

Patient No.	Age (yrs)	Prior therapy*	Mitomycin dose (mg)	Chest x-ray	Clinical course
1	62	Hexa-CAF, cisplatin, ip MTX, 5-FU	41	Diffuse interstitial infiltrates	Mild hypoxemia (PO_2 = 64) and a reduction in diffusion capacity (56% of predicted); gradual improvement in symptoms and chest x-ray findings; autopsy 4 mos after cessation of mitomycin revealed mild pulmonary fibrosis in the apices of the lung
2	30	CHEX-UP	68	Diffuse interstitial infiltrates	Severe reduction in diffusion capacity (24% of predicted); clearing of chest x-ray and symptoms after mitomycin stopped; autopsy 5 mos later showed bilateral moderate pulmonary fibrosis
3	51	PAC, MTX 5-FU, ACA	90	Diffuse interstitial infiltrates	Progressive respiratory failure; transient response to steroids followed by overwhelming bacterial pneumonitis; autopsy showed interstitial fibrosis, proliferation of type II cells, and focal intra-alveolar fibrosis
4	60	PAC, ACA	45	Pleural effusions, venous congestion, pulmonary edema	Died of acute respiratory failure with pulmonary edema 3 wks after last dose; no autopsy
5	58	PAC, MTX, 5-FU, HMM	93	Bilateral effusions and reticulonodular infiltrates	Transbronchial lung biopsy nondiagnostic; chest x-ray cleared after mitomycin stopped and steroid was administered

*Hexa-CAF = hexamethylmelamine (HMM); cyclophosphamide, methotrexate (MTX), and 5-FU; CHEX-UP = cyclophosphamide, HMM, 5-FU, and cisplatin; PAC = cisplatin, doxorubicin, and cylcophosphamide; and ACA = aclarubicin.

edema. Autopsies were performed in three patients. In two of these patients, respiratory symptoms and chest x-ray findings had gradually improved after cessation of mitomycin and these patients died of refractory ovarian cancer 4 and 5 months after mitomycin therapy. Both patients, one of whom was only 30 years old, had evidence of pulmonary fibrosis in the lung with the pathologic appearance consistent with, but not diagnostic of, drug-induced injury. The patient with progressive respiratory failure had a histologic appearance in her lungs identical to that described for mitomycin-induced lung damage with interstitial fibrosis and proliferation of type II pneumocytes (6,7).

DISCUSSION

The lack of objective responses in the 11 patients who received more than one cycle of MVP suggests that it is not an active regimen in patients with refractory ovarian cancer. Mitomycin was most likely responsible for the high incidence (39%) of pulmonary toxicity observed in this trial (6–9). However, the rapidity of onset of symptoms after initiation of MVP therapy, the low total dose of MVP, and the high incidence of lung toxicity are not typical of previous reports on mitomycin lung toxicity. It is possible that the prior drug exposure of these patients with ovarian cancer had resulted in subclinical pulmonary damage which made the patients more susceptible to the toxic effects of mitomycin. Patients with refractory ovarian cancer being considered for therapy with mitomycin should have pulmonary function tests to determine if they have a decrease in diffusion capacity which may increase the risk for mitomycin lung toxicity.

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Effects of Cimetidine, Antipyrine, and Pregnenolone Carbonitrile on Misonidazole Pharmacokinetics[1]

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We investigated the effects of cimetidine, antipyrine, and pregnenolone carbonitrile on the pharmacokinetics of misonidazole (MISO) in mice. Antipyrine did not induce MISO demethylation. Considerable induction was seen with pregnenolone carbonitrile, but it was not superior to phenobarbital. Cimetidine inhibited MISO demethylation and increased its half-life and area under the curve. We suggest that patients receiving cimetidine and MISO have their MISO pharmacokinetics carefully monitored. [Cancer Treat Rep 67:723–725, 1983]

Neurotoxicity limits the dose of the radiosensitizer and chemosensitizer misonidazole (MISO) in man (1). Patients (mainly with glioma) receiving both the anticonvulsant phenytoin and the steroid dexamethasone have a reduced incidence of neuropathy (2). Although anticonvulsants increase the clearance of MISO by inducing enzymatic demethylation (2,3), they have no protective effect when given alone,[3] whereas dexamethasone alone is protective (4).

Most patients with glioma receive dexamethasone to control cerebral edema, and it may now be given to other patients to prevent MISO neurotoxicity. A side effect of dexamethasone is gastric ulceration, for which cimetidine is often given. Since cimetidine inhibits drug-metabolizing enzymes (5) and diminishes renal clearance (6), we were concerned that it might inhibit MISO elimination, thereby increasing drug exposure. Here we report the effects of cimetidine on the pharmacokinetics of MISO and its desmethylmetabolite, DEMIS, in mice. We also describe the effects of alternative enzyme inducers, namely, antipyrine, which is an excellent enzyme inducer in man (7), and the catatoxic steroid pregnenolone carbonitrile (PCN), which is highly potent as an enzyme inducer (8) and as a protector against drug toxicity (9) in experimental animals.

MATERIALS AND METHODS

BALB/c male mice were obtained from OLAC (Bicester, UK) and used when they weighed 20–30 g. Drugs were obtained from the following sources: MISO and DEMIS, Roche (Welwyn Garden City, UK); cimetidine, Smith Kline and French (Welwyn Garden City, UK); antipyrine, Sigma (Poole, UK); PCN, Searle (Chicago, IL); phenobarbital, BDH (Poole, UK); and pentobarbital (Sagatal), May and Baker (Dagenham, UK).

All injections were ip in a volume of 0.01 ml/g, except for cimetidine (0.02 ml/g) and high-dose (1 g/kg) MISO (0.04 ml/g). Cimetidine (250 mg) was dissolved in 1 N HCl (1.1 ml), and 0.1 N NaOH (2 ml) was added; addition of sterile water (6.9 ml) and 0.85% saline (15 ml) followed. PCN (and sometimes phenobarbital) was made up in saline containing 0.5% Tween 80. All other agents were prepared in saline or Hanks' salt solution.

Cimetidine was given 30 minutes before nitroimidazoles or pentobarbital. Other test agents were given daily on Days 1–5, with the nitroimidazoles or pentobarbital given on Day 7 (10). Controls received appropriate vehicles.

Blood was obtained from the tail or by cardiac puncture. Analysis of MISO and DEMIS in blood was by reverse-phase, high-performance liquid chromatography (11).

RESULTS

Cimetidine

When 200 mg/kg of cimetidine was given to mice 30 minutes before 30 mg/kg of pentobarbital, sleep-time (± 2 SE) increased from 35 (21–57) to 98 (79–122) minutes ($0.02 > P > 0.01$). As the sleep-time in control mice given

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45 mg/kg was 94 (71-123) minutes, the dose-modification factor for cimetidine enhancement was 1.5. Thus, cimetidine inhibited drug-metabolizing enzymes in our mice.

Three independent experiments were carried out in which 200 mg/kg of cimetidine was given 30 minutes before 100 mg/kg of MISO or DEMIS. The half-life for MISO elimination was extended 38%-69% (table 1A), and the area under the curve ($AUC_{0-\infty}$) correspondingly increased (data not shown). Inhibition of MISO demethylation to form DEMIS was confirmed by the lower metabolite concentrations in cimetidine-treated mice (table 1A). Peak MISO concentrations and the volume of distribution (Vd_{extrap}) were both unaffected by cimetidine (data not shown).

The metabolite DEMIS is eliminated predominantly by renal clearance (10). Results of similar experiments with injected DEMIS are shown in table 1B. Cimetidine did not increase the half-life for DEMIS clearance, and in one experiment the half-life was actually reduced.

Antipyrine

Antipyrine was given daily on Days 1-5 at 50, 100, 200, 300, or 400 mg/kg, with 100 mg/kg of phenobarbital included as a positive control. MISO (1 g/kg) was given on Day 7. In each of two independent experiments, no changes in MISO pharmacokinetics were seen at any antipyrine dose, whereas phenobarbital reduced the half-life from 1.90 (1.54-2.47) hours to 1.31 (1.10-1.59) hours ($0.02 > P > 0.05$).

Pregnenolone Carbonitrile

PCN was tested at 10 and 100 mg/kg/day and compared with 80 mg/kg/day of phenobarbital. Results obtained from two independent experiments were very similar, and data from one are shown in table 2. More rapid clearance was seen at both doses of PCN, with the effect being more marked at the higher dose. However, the reduction in MISO half-life and the increased metabolite concentrations were no greater than with phenobarbital.

DISCUSSION

Although antipyrine is a good inducer of drug-metabolizing enzymes in man (7), we found no induction of MISO metabolism in the mouse. Induction of demethylation and increased clearance of MISO were seen with the catatoxic steroid PCN, but this was no greater than with phenobarbital. Thus, these agents are unlikely to be useful as alternative inducers of MISO metabolism for clinical use.

The results obtained with cimetidine show that this agent can inhibit MISO demethylation and increase its half-life and area under the curve. Despite its reported effects on renal clearance (6), cimetidine did not alter the pharmacokinetics of DEMIS. It seems prudent to advise that patients receiving cimetidine and MISO should have their MISO pharmacokinetics carefully monitored. Similar precautions might also be appropriate for newer nitroimidazoles such as Ro 03-8799 and SR 2508.

TABLE 1.—Effects of 200 mg/kg of cimetidine on the pharmacokinetics of MISO (100 mg/kg) or DEMIS (100 mg/kg) in BALB/c mice *

	Experiment		
	1	2	3
A. MISO			
MISO half-life (mins)†			
Control	34 (28-44)	33 (30-38)	33 (28-40)
Cimetidine pretreatment	47 (41-56)‡	56 (49-64)§	53 (40-58)¶
Peak DEMIS metabolite concentration (μg/ml) * *			
Control	20.7 ± 2.4	20.2 ± 4.5	13.2 ± 3.8
Cimetidine pretreatment	15.6 ± 1.9¶	14.8 ± 1.9	8.4 ± 2.4
B. DEMIS			
DEMIS half-life (mins)†			
Control	56 (50-63)	58 (48-74)	67 (53-92)
Cimetidine pretreatment	60 (43-101)	42 (35-51)‡	59 (40-113)

* In each experiment, 3-4 time points were included, with 4 mice/time point for both treated and control groups.
†95% confidence limits in parentheses.
‡$0.05 > P > 0.02$.
§$P < 0.001$.
¶$0.02 > P > 0.01$.
* * ± 2 SE (n = 4).

TABLE 2. —Effects of PCN (10 or 100 mg/kg × 5) on the pharmacokinetics of MISO (1 g/kg) in BALB/c mice*

Pretreatment	MISO half-life† (hrs)	Peak DEMIS metabolite concentration‡ (μg/ml)
Saline	1.59 (1.41–1.83)	37.9 ± 12.6
Tween 80 (0.5%)	1.60 (1.37–1.93)	49.9 ± 20.2
PCN (10 mg/kg)	1.31 (1.18–1.46)	40.8 ± 12.0
PCN (100 mg/kg)	0.98 (0.90–1.46)§	78.2 ± 3.8¶
Phenobarbital (80 mg/kg)	1.00 (0.83–1.27)**	88.1 ± 43.4

*In each experiment, 3 time points were included, with 5 mice/time point for both treated and control groups.
†95% confidence limits in parentheses.
‡ ± 2 SE (n = 5).
§$0.01 > P > 0.001$ compared to Tween 80 control.
¶$0.05 > P > 0.02$.
**$P < 0.001$.

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Combination Chemotherapy With Cisplatin, Etoposide, and Vindesine in Non-Small Cell Lung Carcinoma: A Clinical Trial of the EORTC Lung Cancer Working Party[1,2,3]

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A combination of cisplatin (60 mg/m² on Day 1), etoposide (120 mg/m² on Days 3, 5, and 7), and vindesine (1.5 mg/m² on Days 1 and 7), repeated every 3 weeks, was administered to 73 patients with non-small cell bronchogenic carcinoma. After two full courses, the results could be evaluated in 62 patients, 25 (40.3%) of whom responded (five complete responses, 20 partial responses). The median survival for the responding patients (12 months) was significantly superior ($P = 0.02$) to that of the nonresponding patients. There were three early toxic deaths from sepsis associated with granulocytopenia, and seven patients presented peripheral neuropathy. Although active in non-small cell bronchogenic carcinoma, the combination of cisplatin, etoposide, and vindesine does not appear to be superior to cisplatin-etoposide or cisplatin-vindesine when our previous experience and the results reported from other institutions are considered. [Cancer Treat Rep 67:727–730, 1983]

Among the newer chemotherapeutic compounds, cisplatin has been investigated in advanced non-small cell lung cancer (1,2); moderate activity has been reported. On the other hand, the combinations of cisplatin-vindesine (3) or cisplatin-etoposide (4) were found to be remarkably effective in advanced non-small cell carcinoma of the lung. For this reason, the European Organization for Research on Treatment of Cancer Lung Cancer Working Party (Belgium) undertook a clinical phase II study in advanced non-small cell carcinoma using a three-drug combination containing cisplatin, etoposide, and vindesine to define objective response and survival rates further.

PATIENTS AND METHODS

Seventy-three patients from 16 Belgian institutions, with histologically proven non-small cell bronchogenic carcinoma, were entered in the study. All patients had measurable tumors (defined tumor margins allowing measurement with calipers) or evaluable disease (lesions visible and evaluable without precise measurement of size).

The therapeutic regimen consisted of cisplatin (60 mg/m² on Day 1), etoposide (120 mg/m² on Days 3, 5, and 7), and vindesine (1.5 mg/m² on Days 1 and 7). The therapeutic schedule was routinely repeated after 3

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weeks, but, for several patients, an additional week was allowed before repeating treatment because of hematologic and/or psychologic considerations. Dose reductions were made according to the hematologic (for etoposide and vindesine) and renal (for cisplatin) toxicity of the preceding course of therapy.

The response evaluation was made at 6–7 weeks and data to document it were presented and discussed at monthly meetings of the Group. Complete remission (CR) was defined as the disappearance of all known disease for at least 4 weeks, including a negative fibrobronchoscopy with multiple negative biopsies. Partial response (PR) was defined as a $\geq$ 50% decrease in the total tumor size, with no lesions appearing or progressing. Stable disease (when the estimation of tumor reduction was < 50%), progressive disease (defined as a $\geq$ 25% increase in tumor size), and toxic death (defined as death occurring during the first 6 weeks due to drug toxicity) were considered failures. However, many patients with stable disease at 6 weeks received one or more additional courses. Therapy was discontinued at the time of progression and/or excessive morbidity in partial responders. Ten courses were given to the patients achieving CR.

RESULTS

All of the 73 registered patients were given a minimum of one course of therapy; however, 11 patients could not be evaluated at 6 weeks for tumor response because of death either from sepsis related to severe granulocytopenia (toxic deaths: three patients) or from other causes unrelated to therapy (nontoxic early deaths: eight patients).

The median age was 62 years (range, 32–75); the median performance status was 70% (range, 60%–100%). Lesions were measurable in 39 patients (63%) and were evaluable in 23 (37%).

Squamous cell carcinoma was present in 38 (61%) patients, adenocarcinoma was found in 17 (27%), large cell carcinoma was found in four (6.5%), and mixed types were found in three (5%). Locoregional disease (limited to the chest and supraclavicular nodes) was diagnosed in 25 (40%) patients and disseminated disease, in 37 (60%). Seven patients (11%) had received prior radiotherapy but none had received chemotherapy alone; two patients had received both radiotherapy and chemotherapy.

As indicated in table 1, the response rate in patients evaluable after two full courses of therapy was 40% (25 of 62 patients), with five CR. The response rate was higher in patients with squamous cell carcinoma (44%) than in those with adenocarcinoma (18%). The overall response rates were 38% (25 of 65 patients), if the three patients with toxic death were taken into account, and 34% (25 of 73 patients), if all of the eligible patients were considered. We found a similar response rate whether or not the patients had received prior therapy; the response rates were 44% and 40%, respectively; the difference is not statistically significant. In regard to disease extension, 32% of the patients (eight of 25) with locoregional disease responded and 46% of the patients (17 of 37) with disseminated disease responded; the difference is not statistically significant. PR were observed after one or two courses of therapy; no patient went into remission later than after the second course.

The median duration of PR was 29 weeks. The duration of CR was 17 months for one patient who died from cerebral metastases; two other patients are still in CR at 23 and 15 months; and two patients relapsed 11 and 9 months after the start of therapy (both are alive at 15 and 18 months, respectively).

Platelet count toxicity was not important in the present series; in 80% of the courses, the platelet count remained $\geq$ 100,000/mm^3. In 56% of the courses, the wbc count dropped to < 3000/mm^3; in 29%, it dropped to < 2000/mm^3. Six patients (12%) had wbc counts < 1000/mm^3.

Because of the dose reduction, the toxicity of multiple courses of therapy could not be evaluated; however, no cumulative toxicity was observed with respect to platelet and wbc counts.

As indicated in table 2, most patients developed alopecia and had gastrointestinal toxic effects consisting of nausea and vomiting during therapy. During the first two courses of chemotherapy, 11 patients developed diarrhea and five presented fever during episodes of leukopenia; infection could be microbiologically documented in one of them. The four other patients had

TABLE 1.—Response to chemotherapy

Type	No. of patients		
	Total	CR	PR
Squamous cell carcinoma	38	2	15
Adenocarcinoma	17	3	1
Large cell carcinoma	4	0	1
Mixed types	3	0	3
Total	62	5 (8%)	20 (32%)

febrile episodes without documented infection. As previously mentioned, three patients died (toxic deaths) from sepsis associated with granulocytopenia before receiving the second course of therapy.

Hearing loss was observed in two patients and peripheral neuropathy, sometimes severe, was observed in seven patients during the first two courses of chemotherapy. Renal toxicity, a potential complication of cisplatin, was seen in only three patients who had a transient and moderate increase in serum creatinine. Stomatitis was observed in five patients and was never severe. There was no indication that cumulative toxicity occurred. Overall, therapy had to be discontinued because of adverse reactions (mainly nausea and vomiting) in nine patients.

Responding patients survived longer than the nonresponders. Median survival was 12 months for responders and 7 months for nonresponders; the difference is statistically significant ($P = 0.02$). The overall survival is indicated in figure 1.

TABLE 2.—Nonhematologic toxicity

	No. of patients after course—					
	2	3	4	5	6	7
Evaluable	65	25	17	11	9	8
Toxic effect						
Toxic death*	3 (5%)	0	0	0	0	0
Alopecia	44 (68%)	18	12	8	6	4
Nausea and/or vomiting	52 (80%)	15	12	6	6	4
Diarrhea	11 (17%)	5	3	2	0	0
Fever	4 (6%)	2	0	0	0	0
Documented infection	1 (1.5%)	0	0	0	0	0
Hearing loss	2 (3%)	1	0	0	0	0
Neuropathy	7 (11%)	0	0	0	0	0
Nephrotoxicity	3 (5%)	0	0	0	0	0
Stomatitis	5 (8%)	1	0	0	0	0
Discontinuation of therapy	9 (14%)	0	0	0	0	0

*Occurred before the 2nd course of chemotherapy.

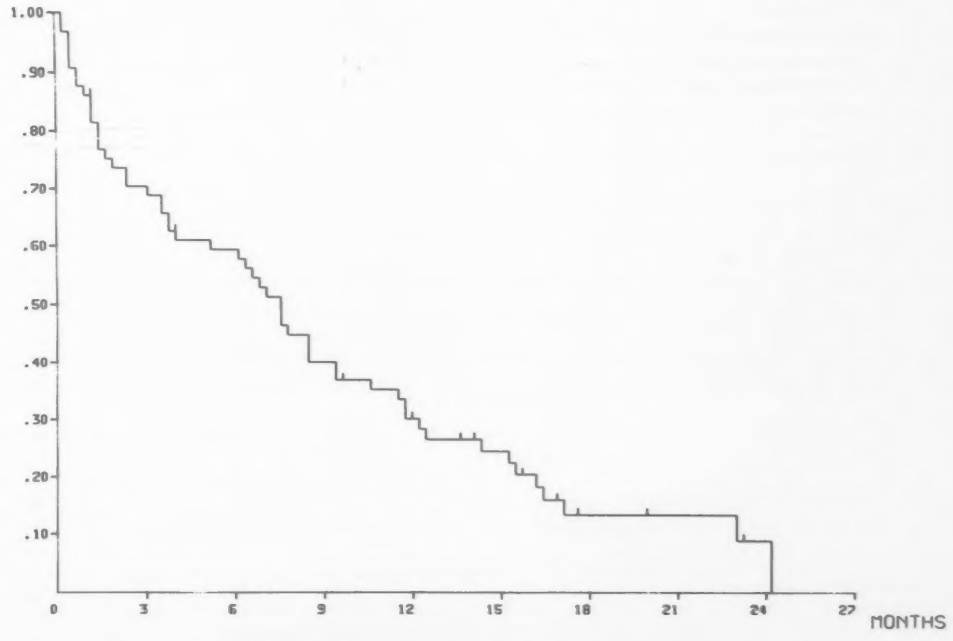

FIGURE 1.—Survival from the start of therapy for all patients.

DISCUSSION

Gralla et al (3) reported a response rate of 43% with the cisplatin-vindesine combination; they also observed that a high dose of cisplatin (120 mg/m²) was superior to a lower dose (60 mg/m²) in median duration of response (12 vs 5.5 months) and in median survival for responding patients (21.7 vs 10 months). Our Group (4) has reported similar results with cisplatin (60 mg/m²) and etoposide in squamous cell carcinoma and adenocarcinoma of the lung, with an overall response rate of 37%. The median survival of responding patients in that series was 16 months.

The addition of vindesine to the combination of cisplatin-etoposide was not associated here with an improved response rate as compared to the results reported for cisplatin-vindesine or cisplatin-etoposide. An overall response rate of 34% (25 of 73 patients) was observed in the present series, while a 37% rate (36 of 87 patients) was observed in our previous study with cisplatin-etoposide; the median survival times for the responding patients were 12 and 16 months, respectively, in these two consecutive investigations.

Whether our results may be improved by the use of higher doses of vindesine must be considered. However, the neurotoxicity of the three-drug regimen (cisplatin, etoposide, and vindesine) as used here was higher than that of cisplatin and etoposide without vindesine. Neurotoxicity occurred here in seven of 62 patients (11%) but had not been seen in our previous study.

Why the addition of vindesine to cisplatin and etoposide did not increase the response rate remains unclear. Similarly, it has been shown recently by Kelsen et al (5) that the addition of doxorubicin and/or cyclophosphamide did not secure any advantage for the cisplatin-vindesine combination. There is no evidence that, in our study, the drug dose reduction because of adverse reactions reached a level that would explain our results. Another theoretic possibility is that vindesine antagonizes the combination of cisplatin-etoposide. There are no data to substantiate this at the present time.

To conclude, our results suggest that the addition of vindesine to cisplatin plus etoposide is not indicated for the treatment of non-small cell bronchogenic carcinoma. The response rate and the duration of survival are not superior to those obtained with the combination of cisplatin-vindesine (3) or cisplatin-etoposide (4).

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Enhancement of Rat Heart Microsomal Lipid Peroxidation Following Doxorubicin Treatment In Vivo[1]

Edward G. Mimnaugh,* Michael A. Trush, and T. E. Gram[2]

Cardiac microsomes from rats injected with doxorubicin (5 mg/kg ip × 3) were susceptible to dramatically enhanced NADPH-dependent lipid peroxidation in the presence of doxorubicin in vitro (18-fold increases over control peroxidation). These results suggest that doxorubicin treatment in vivo caused biochemical alterations which predisposed cardiac microsomal membranes to attack by anthracycline free radical-generated oxyradicals. [Cancer Treat Rep 67:731–733, 1983]

Numerous complex biochemical changes accompany the degenerative cardiomyopathy which may result from aggressive anthracycline therapy (1). Although not yet verified in humans, lipid peroxidation resulting from anthracycline free radical and secondary oxyradical formation has been implicated as a possible biochemical mechanism which contributes to the pathogenesis of anthracycline-caused cardiac membrane system damage (2–4). Several recent reports (4,5) have demonstrated that, although rat liver microsomes were highly susceptible to anthracycline-enhanced lipid peroxidation, rat heart microsomes were refractory to peroxidation, unless the cardiac microsomes were isolated from α-tocopherol-deficient animals, in which case anthracyclines stimulated peroxidation as much as tenfold. However, rats fed an α-tocopherol-adequate diet developed chronic doxorubicin cardiomyopathy and congestive heart failure similar to other species (6). To reconcile this apparent contradiction and to further test the lipid peroxidation hypothesis of anthracycline cardiotoxicity, we conducted the experiments presented in this report, which suggest that chronic doxorubicin treatment causes biochemical alterations which predispose the myocardium to peroxidative damage by subsequent exposure to doxorubicin.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), 250–350 g, were fed Purina rodent chow and water ad libitum. Doxorubicin (obtained from the Division of Cancer Treatment, National Cancer Institute) was dissolved in 0.9% NaCl and administered ip at a dose of 5 mg/kg three times, at 6, 4, and 1 days before rats were killed; control animals were injected ip with an equivalent volume of 0.9% NaCl solution. A separate group of rats were fed an α-tocopherol-deficient diet (< 0.1 μg α-tocopherol/g, ICN, Cleveland, OH) for 6–8 weeks. Biochemicals were obtained from Sigma Chemical Co (St. Louis, MO).

Animals were killed by cervical fracture. The hearts were removed and rinsed, the ventricles were homogenized in cold 150 mM KCl-50 mM Tris-HCl buffer (pH 7.4), and microsomes were isolated by differential centrifugation as previously described (7). Rat heart microsomes were incubated at 37°C with an NADPH-generating system in KCl-Tris-HCl buffer (pH 7.4) under an oxygen atmosphere, and lipid peroxidation was quantified by the 2-thiobarbituric acid method (8), as described in detail (4,5). Results are expressed as nmols of malonaldehyde equivalents/mg of microsomal protein/time indicated, and mean values which were statistically significant at $P < 0.05$ ($n = 4–8$), as determined by Student's t test, are indicated. Protein concentrations were measured by the method of Lowry et al (9) with bovine serum albumin as the standard.

RESULTS

Incubating heart microsomes from control rats with the NADPH-generating system for as long as 2 hours

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produced negligible lipid peroxidation in the absence or presence of doxorubicin (table 1). In contrast, when heart microsomes from doxorubicin-treated rats were used, considerable quantities of malonaldehyde were produced in the presence of doxorubicin added in vitro, resulting in an 18-fold enhancement of lipid peroxidation (table 1). These results suggest that doxorubicin administration caused some biochemical alteration, such that cardiac microsomes from doxorubicin-treated animals were sensitized to a subsequent doxorubicin-caused reactive oxyradical challenge in vitro.

The increased lipid peroxidation in cardiac microsomes from doxorubicin-treated animals was apparently not due to an elevation in NADPH-cytochrome *c* (P-450) reductase activity, which was 27 ± 4 and 22 ± 2 nmols of cytochrome *c* reduced/mg of protein/minute for microsomes from control and doxorubicin-treated rats, respectively. Doxorubicin (100 μM) had no effect on the measurement of microsomal cytochrome *c* reductase activity.

As shown in table 2, cardiac microsomes from α-tocopherol-deficient rats were susceptible to considerable endogenous NADPH-dependent lipid peroxidation, and in contrast to cardiac microsomes from rats fed a control α-tocopherol-adequate diet (30 IU of α-tocopherol acetate/lb of diet), peroxidation was enhanced more than fivefold by doxorubicin. Compared to the negligible amount of peroxidation observed in cardiac microsomes from control rats in the presence of doxorubicin, the magnitude of peroxidation produced by doxorubicin in α-tocopherol-deficient rat heart microsomes was increased 20-fold (table 2). These results indicate that the α-tocopherol status of cardiac microsomes was a significant determinant in controlling lipid peroxidation.

DISCUSSION

Although the precise mechanism(s) by which anthracyclines cause delayed cardiotoxicity remains elusive, it appears that the enzymatic formation of semiquinone free radicals and enhanced reactive oxygen generation may play crucial roles in the pathogenesis of this lesion. Reactive oxyradicals, such as superoxide anion and hydroxyl radical, mediate anthracycline-enhanced peroxidation in vitro of microsomal (4) and mitochondrial (10) membrane lipids. However, if membrane peroxidation does occur in vivo, it is presently unclear how peroxidation contributes to the evolution of the characteristic, delayed anthracycline cardiotoxicity, since lipid peroxidation would be expected to cause early, acute membrane damage.

A reasonable explanation for the delayed onset of cardiotoxicity is that anthracycline treatment progressively alters or overwhelms biologic defenses against reactive oxygen and eventually sensitizes the myocardium to the toxic consequences of subsequent exposure to anthracyclines. In this context, doxorubicin treatment has been

TABLE 1.—Increase in doxorubicin-stimulated rat heart microsomal lipid peroxidation following doxorubicin treatment in vivo

Treatment in vivo	Incubation time (mins)	Lipid peroxidation (nmols of malonaldehyde equivalents/mg of protein/time point)	
		Endogenous	Doxorubicin (100 μM) added in vitro
Control (0.9% NaCl × 3)	60	1.3 ± 0.1	1.2 ± 0.7
	120	1.2 ± 0.5	1.6 ± 1.1
Doxorubicin (5 mg/kg × 3)	60	0.7 ± 0.3	13.4 ± 3.5*,†
	120	2.4 ± 0.6	42.6 ± 8.7*,†

*Significantly increased compared to values in the absence of doxorubicin in vitro, mean ± SD, n = 3–6.
†Significantly increased compared to values in the presence of doxorubicin in vitro with microsomes from the control group.

TABLE 2.—Doxorubicin-enhanced lipid peroxidation in heart microsomes from rats fed an α-tocopherol-deficient diet

Treatment	Lipid peroxidation (nmols of malonaldehyde equivalents/mg of protein/60 mins)	
	Endogenous	Doxorubicin (100 μM)
Control diet	0.9 ± 0.4	1.6 ± 0.9
α-Tocopherol-deficient diet	5.9 ± 0.7*	33.2 ± 2.2*,†

*Significantly increased compared to values obtained with microsomes from animals fed the control diet, mean ± SD, n = 4–8.
†Significantly greater than values measured in the absence of doxorubicin.

shown to cause a decrease in cardiac glutathione peroxidase activity (11) and to deplete cardiac glutathione levels (12); both of these observations are consistent with the concept that doxorubicin initiated an oxidative stress in the heart. Another important defense against oxyradical toxicity is the membrane antioxidant and free radical scavenger, α-tocopherol. The vital role for α-tocopherol in controlling membrane lipid peroxidation has recently been demonstrated by Kornbrust and Mavis (13), and it is possible that the cardiac sarcoplasmic reticulum and mitochondrial membrane α-tocopherol status is a significant determinant in the development of anthracycline cardiotoxicity. Indeed, α-tocopherol administration significantly diminished the acute toxicity and mortality of doxorubicin in mice (14), and α-tocopherol in vitro nearly abolished doxorubicin-stimulated microsomal lipid peroxidation (4). Moreover, anthracyclines exacerbated lipid peroxidation in heart microsomes from α-tocopherol-deficient rats, but in contrast, they had no effect on the minuscule amount of peroxidation which occurred in cardiac microsomes from control rats (5). These results suggest that rat heart microsomes are normally resistant to peroxidation and that the relatively high level of α-tocopherol found in heart membranes contributes to this resistance.

The dramatic (18-fold) increase in lipid peroxidation in cardiac microsomes from rats treated with multiple injections of doxorubicin, and the greatly enhanced peroxidation which occurred in cardiac microsomes from α-tocopherol-deficient rats in the presence of doxorubicin, strongly suggest that doxorubicin may cause a transient α-tocopherol-deficient state in cardiac membranes. The inactivation of this important membrane protection against oxyradicals would unbalance the multiple, interdependent cellular defenses permitting anthracycline-activated oxyradicals to attack unsaturated lipid targets and propagate lipid peroxidation leading to membrane damage and cytotoxicity.

We are currently investigating the effects of chronic doxorubicin treatment on cardiac microsomal and mitochondrial membrane α-tocopherol levels, and we are extending these preliminary findings to include an evaluation of the dose-dependency and time-course of the effects of doxorubicin in vivo on the peroxidation of cardiac membrane lipids determined in vitro. These results will be reported in a subsequent publication.

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Thymidine and 5-FU: A Phase II Pilot Study in Colorectal and Breast Carcinomas[1]

Cary A. Presant,* Phyllis Multhauf, Leonard Klein, Clara Chan, Fong-Fu Chang, Gilbert Hum, Robert Joseph, Richard Opfell, Steven Lemkin, Thomas Shiftan, and David Plotkin[2]

In experimental tumor models, thymidine (TdR) is synergistic with 5-FU (1,2). Phase I studies (3,4) suggested some response in colorectal carcinoma.

We therefore performed a pilot phase II trial in 27 patients who were not previously resistant to 5-FU. The median performance status was 80% and the median age was 60 years. All patients had normal hematologic, renal, and hepatic functions.

All patients received TdR at a dose of 9 g/m²/infusion over 30 minutes. One hour later, 5-FU was administered by bolus injection. The initial dose for 5-FU was 325 mg/m², but when granulocytopenia (< 1000 cells/mm³)

TABLE 1.—Patient characteristics, response (by site), and drug toxicity

	No. of patients (%)	No. of responses (duration)		
		PR	Stable disease	Disease progression
Primary site				
Colorectal	9	2 (11, 9 wks)	2 (11, 8 wks)	5
Lung	6	0	3 (24, 29, 8 wks)	3
Breast	5	1 (7 wks)	1 (17 wks)	3
Head and neck	3	0	2 (8, 4+ wks)	1
Prostate	3	0	0	3
Ovary	1	0	1 (9 wks)	0
Toxicity*				
Granulocyte count nadir (cells/mm³)				
>1900	6 (21)			
1000–1900	5 (10)			
<1000	18 (62)†			
Platelet count nadir (cells/mm³)				
>130,000	17 (59)			
50,000–130,000	6 (21)			
< 50,000	3 (10)			
Nausea or vomiting				
None	16 (55)			
Mild to moderate	9 (31)			
Severe	2 (7)			
Stomatitis	5 (17)			
Diarrhea	5 (17)			
CNS	3 (10)			

*Toxicity observed during 1st course of therapy.
†2 episodes of septicemia (at 325 mg/m² only).

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was observed in all six patients at this dose, the initial dose was reduced to 225 mg/m². Therapy was repeated every 28 days until evidence of tumor progression. Partial response (PR) was defined as a > 50% decrease in tumor area. The dose of 5-FU was reduced by 50 mg/m² if severe toxicity was observed.

Only three responses were achieved (table 1); all were of short duration (11, 9, and 7 weeks). The dose-limiting toxic effect was myelosuppression. Although only 11% of patients had PR, 62% of patients had granulocytopenia < 1000 cells/mm³. Granulocytopenia was more severe than thrombocytopenia. Gastrointestinal toxicity was not marked. The previously reported high incidence of neurotoxicity of this regimen (5) was not confirmed in our patients. Only three patients had neurotoxic effects: one each with incoordination, disequilibrium, and confusion and ataxia.

DISCUSSION

Although only two of nine (22%) patients with colorectal carcinoma and one of five (20%) patients with breast carcinoma had PRs, these response rates are similar to that of 5-FU alone, and the responses were of only short duration. However, severe toxicity was observed, which required decreasing the previously recommended dosage of 5-FU in this regimen (4) to initial levels of only 225 mg/m² every 28 days. We conclude that these minimal results do not warrant further trials in these tumor types, unless other types of metabolic modulation are also included, or unless the schedule of administration is modified.

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Phase II Evaluation of Dianhydrogalactitol in the Treatment of Advanced Endometrial Adenocarcinoma: A Gynecologic Oncology Group Study[1,2]

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In a broad phase II study by the Gynecologic Oncology Group, dianhydrogalactitol (DAG), a hexitol diepoxide which has been shown to have antitumor activity (1), was evaluated in advanced gynecologic tumors. Some activity of this agent against squamous cell carcinoma of the cervix and ovarian epithelial carcinoma had been identified (2).

Twenty-one patients with advanced or recurrent endometrial carcinoma not amenable to other, more standard therapy were entered in the protocol. The following criteria were required for entry in the study: measurable disease and histologic confirmation of tumor, recovery from prior therapy, adequate blood cell counts (granulocyte count $\geq 2000/\mu l$ and platelet count $\geq 100{,}000/\mu l$), and adequate renal and hepatic functions. One patient was considered ineligible on review since tumor could not be documented, and three patients were never treated. Seventeen patients were evaluable for toxicity and response. Patient characteristics are presented in table 1. The median age of patients was 63 years (range, 58–78). The initial DAG dose was 60 mg/m² each week, increasing after four courses to 75 mg/m² if there were no adverse hematologic effects. Of nine patients who received more than four courses, none received this dose escalation.

There were no complete tumor responses and only one partial response (duration, 1 month) among 17 evaluable patients. Eight patients demonstrated marked disease progression within 4 weeks of starting therapy and eight patients had stable disease (median duration, 4.5 months). Patients with increasing disease had significantly shorter survival when compared to other patients (4.4 vs 7.4 months, $P = 0.05$).

Adverse effects, comparable to our previous experience with this drug (2), are summarized in table 1. The median nadir for those patients with wbc count toxicity was $3600/\mu l$ (range, 3100–3900) and the median nadir for those with platelet count toxicity was $100{,}000/\mu l$ (range, 49,000–142,000). The dose regimen was the same as that utilized in our previous experience (2). These patients, who were predominantly older and more heavily pretreated, had fewer severe adverse effects. Since eight of 17 patients had tumor progression within the first 4 weeks, the mean number of courses of treatment given was fewer (5.0).

In conclusion, DAG at the dose and schedule tested had an acceptable level of adverse effects even in this group of patients with extensive prior treatment and advanced median age. We believe that this study represents an adequate trial of the drug. DAG at the dose and schedule tested appears to have negligible activity in the treatment of patients with advanced, endometrial carcinoma whose disease has progressed on both hormonal therapy and chemotherapy.

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TABLE 1.—Characteristics of study patients

Characteristic	No. of patients
Histologic grade*	
1	4
2	8
3	5
Prior therapy	
Surgery	15
Radiotherapy	14
Hormonal therapy	14
Chemotherapy	17
Prior chemotherapy	
Doxorubicin ± cyclophosphamide ± 5-FU	11
Melphalan + 5-FU	8
Etoposide	7
Cisplatin	2
Dacarbazine	2
No. of prior chemotherapy trials	
1	5
2	11
3	1
Performance status†	
0	0
1	8
2	9
Site of disease	
Pelvic	6
Extrapelvic	11
Response	
Complete	0
Partial	1
Stable disease	8
Progression	8
Wbc count toxicity†	
0	10
1	7
Platelet count toxicity†	
0	9
1	4
2	3
3	1
Gastrointestinal toxicity†	
0	8
1	4
2	5
Renal toxicity†	
0	10
1	5
2	1
3	1

*International Foundation of Gynecology and Obstetrics criteria.
†Gynecologic Oncology Group grade (2).

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Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone (CHOP) in Patients With Advanced Hodgkin's Disease: A Southwest Oncology Group Phase II Study[1,2]

Thomas P. Miller,[3] Stephen E. Jones,[3] and John M. Rainey[4,*]

Since the initial description of successful treatment of patients with Hodgkin's disease (HD) by DeVita et al (1), combination chemotherapy with mechlorethamine, vincristine, procarbazine, and prednisone (MOPP) has become the standard of systemic therapy for this disease. Other chemotherapeutic agents including doxorubicin have activity in HD (2). Recently, Bonadonna et al (3) have shown that the doxorubicin-containing regimen ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) is an effective alternative to MOPP. For non-Hodgkin's lymphomas the doxorubicin-containing regimen CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) has proven superiority over non-doxorubicin-containing combinations in terms of response rate and survival duration (4). In this exploratory study the effectiveness of CHOP to induce complete and durable remissions in patients with previously untreated HD was tested.

Previously untreated patients with biopsy-proven stage III or IV HD were eligible for study. Treatment consisted of CHOP in a schedule previously described (4) and administered at 3-week intervals for eight courses. No maintenance therapy was given to patients achieving a complete response (CR). Patients achieving a partial response (PR) after eight cycles of CHOP, or developing progressive disease while on study, or developing recurrent disease after discontinuation of treatment were then given standard MOPP chemotherapy (1). Survival was calculated from the initiation of chemotherapy until death.

Twelve patients were entered in a two-institution pilot study between April 1978 and April 1980; all were eligible for the trial, and all were evaluable. There were ten males and two females, with a median age of 39 years (range, 16–55). The histologic subtypes included nodular-sclerosing HD (eight patients), lymphocyte-depleted HD (two patients), and mixed-cellularity HD (two patients). These patients were in general symptomatic, with a median Karnofsky performance score of 60% (range, 30%–90%).

The clinical characteristics of patients and the results of therapy are summarized in table 1. Objective responses (CR + PR) were observed in all patients. Three patients (25%) achieved CR. The median duration of response for all patients was 13.5 months; the median duration of survival for all patients has not been reached. The median follow-up time is 32 months. The treatment was generally well-tolerated. Seven patients experienced mild to moderate vomiting. All patients experienced some hair loss. Myelosuppression was mild and did not necessitate a dose-rate change for any patient.

MOPP remains the standard of chemotherapy for HD, although recent trials suggest that alternating non-cross-resistant combinations may be more effective (5). In this small exploratory study the CHOP regimen does not appear to offer any advantage with regard to CR rate or survival duration over results obtained using MOPP as initial chemotherapy for advanced HD. The CHOP combination is active as evidenced by the objective response rate of 100% obtained in this study of patients with advanced HD. It is interesting that despite the low CR rate obtained with CHOP, seven of 12 patients are currently free of disease after having received MOPP following tumor-burden reduction with CHOP. Such an observation may warrant a trial of CHOP alternating with MOPP as initial treatment for advanced HD.

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TABLE 1.—Results of treatment with CHOP in HD

HD stage	No. of patients (%)						
	Total	Liver involvement	Bone marrow involvement	Bulky disease*	CR	PR	Survival (mos)
III_2A	3	0	0	1	2 (67)	1 (33)	19, 38+, 48+
III_2B	1	0	0	1	0	1 (100)	17
IVB	8	4	7	4	1 (12)	7 (88)	8, 13, 14+, 34+, 30+, 38+, 45+, 48+
Total	12	4 (33)	7 (58)	6 (50)	3 (25)	9 (75)	

* Bulky disease is defined as a mass > 7 cm in diameter.

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Advanced Adenocarcinoma of the Kidney: Therapy With Lomustine, Vinblastine, Hydroxyurea, and Medroxyprogesterone Acetate and Regression Analysis of Factors Relating to Survival[1,2]

Leonard H. Brubaker,[3,*] Michael B. Troner,[4] and Robert Birch[5]

Of 37 patients with advanced adenocarcinoma of the kidney who were treated with lomustine, vinblastine, hydroxyurea, and medroxyprogesterone acetate (26 had an adequate trial), three had a transient partial response. Patients having a serum albumin $\geq$ 3.8 g/dl or lacking liver involvement survived significantly longer than those not having these characteristics.

Advanced adenocarcinoma of the kidney has remained one of the least responsive tumors to systemic therapy (1).

Because of one complete and one partial response (PR) in a group of eight patients treated with a combination of medroxyprogesterone acetate, lomustine, vinblastine, and hydroxyurea at the University of Louisville,[6] the Southeastern Cancer Study Group (SECSG) undertook a similar study.

METHODS

Eligible patients had measurable disease with a histologic diagnosis of renal adenocarcinoma. One patient was permitted in the study with only a typical appearance on renal arteriogram for the diagnosis. All patients had distant metastatic disease or involvement of adjacent organs, a Karnofsky performance score of $\geq$ 40, and an estimated survival of > 3 months. Prior to the study, informed consent was obtained according to the National Institutes of Health (Bethesda, MD) guidelines.

The treatment regimen was as follows: vinblastine, 5 mg/m^2 iv on Day 1 of each 28-day course; lomustine, 60 mg/m^2 orally on Day 2; hydroxyurea, 400 mg/m^2 orally every 12 hours on Days 3-14; and medroxyprogesterone acetate, 1 g im weekly, starting on Day 1. Therapy continued until there was evidence of disease progression. Response and toxicity were reported according to standard SECSG criteria. Patients underwent baseline routine blood cell counts and chemistry tests.

A Cox regression analysis (2) was performed using the following variables to predict survival: age, sex, initial performance status, prior nephrectomy (yes/no), prior chemotherapy (yes/no), prior radiotherapy (yes/no), length of illness before on-study date, lung involvement (yes/no), liver involvement (yes/no), and hematocrit, serum albumin, alkaline phosphatase, and uric acid values. Of 37 patients, 33 had values for all of these variables, and these were used in the analysis.

PATIENT CHARACTERISTICS

Thirty-seven patients with adenocarcinoma of the kidney were entered in the study; their characteristics are shown in table 1. Twenty-six patients had an adequate trial, including two or more 28-day cycles administered at > 75% of all stipulated drug doses. Six other patients were removed from the study after one cycle because of early disease progression. Five patients were inevaluable because doses of one or more drugs were > 25% too low.

RESULTS

Of the 26 adequately treated patients, three (12%) had a transient PR, but none had a complete response. Their survival from entry in the study is shown at the bottom of table 1. If the six patients with early progression are included, the response rate is 9% (three of 32 patients).

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TABLE 1.—Patient characteristics

Characteristic	
Total No. of patients	37
Males (%)	70
Age in yrs	
Mean	58.4
Range	33-75
Performance status (%)	
Mean	76
Range	40-100
% of patients with—	
Prior therapy	
Nephrectomy	70
Irradiation	27
Chemotherapy	8
Hormonal therapy (Megace)	3
Site of metastases or spread	
Liver	24
Bone	46
Lung	68
Brain	8
Abdomen	22
Supraclavicular nodes	11
History	
No. of wks from—	
Symptoms to entry	
Median	35
Range	3-685
Entry to death (all patients)	
Median	24
Range	3-112+
Entry to death (responders)	29, 43, 61
% of patients who died	95

Of the variables entered into the Cox analysis, only two were found to be important indicators of survival. High values of serum albumin were important (P = 0.0085). Median survival of patients whose initial albumin was $\geq$ 3.8 g/dl was 40 weeks, whereas those whose albumin was less than this had a median survival of 20 weeks (P = 0.0273). Lack of liver involvement was also important (P = 0.0428).

DISCUSSION

These treatment results do not encourage further use of this combination and schedule of drugs. Further efforts against this disease by the SECSG are being directed to phase II studies.

Paulson and co-workers (1) have reported a Cox analysis of 29 patients with renal adenocarcinoma. They found that male sex, serum alkaline phosphatase < 200 units/100 ml, normal hematocrit, and lack of bone metastases were characteristics associated with longer survival in their patients. Thus, we have not been able to confirm their selection of characteristics associated with longer survival.

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Response to: "Is It Ethical Not to Conduct a Prospectively Controlled Trial of Adjuvant Chemotherapy in Osteosarcoma?"

A number of statements and concepts in the article "Is It Ethical Not to Conduct a Prospectively Controlled Trial of Adjuvant Chemotherapy in Osteosarcoma?" (1) cannot go unchallenged.

Until the beginning of the 1970s, patients with osteosarcoma had been treated with surgery, radiation therapy, immunotherapy, and ineffective chemotherapy. This provides a historical group which documents the inevitable dismal prognosis. The cure rate rarely exceeded 20% and was reconfirmed on many occasions. Included in this historical control series are data from the Mayo Clinic.

The validity of historical controls was questioned with a report of a small series of patients from the Mayo Clinic, a short follow-up, and claims of a "natural" improvement in survival over the past 5 years. While there is no apparent explanation for this phenomenon, it is noteworthy that some recent patients at the Mayo Clinic were also treated with investigative adjuvant regimens, including chemotherapy.

A discussion on the Mayo Clinic experience and the use of historical controls is possibly moot in view of the impressive results reported by Rosen. However, these results are unique, and until they can be duplicated, the historical control series still serves as a framework of comparison.

The article cites pulmonary tomography as an improved means for identification of more patients with pulmonary lesions as opposed to those without metastatic disease at diagnosis. However, the yield with pulmonary tomography may be extremely small. In the study by Cohen, the yield was 2.7%. In another study, 12 of 80 patients were identified with pulmonary metastases and in only one of the 12 were metastases evident on tomography and not on the normal chest radiograph (2). Thus, it is unlikely that the majority of patients with normal chest radiographs in the historical control series had undetectable pulmonary disease at diagnosis. Further, it remains to be reported to what extent, if any, the increased sensitivity of computerized tomography will render the use of historical controls invalid.

It is possible that histology, tumor size, location, age, and other factors may influence the outcome of individual patients with osteosarcoma. However, they have less significance when similar or identical results are reported by investigators throughout the world. Notwithstanding, statistical regression methods are available to compensate and adjust for differences when comparing historical control patients with patients in current studies (3).

In contrast to the results with historical controls, adjuvant chemotherapy has had a major impact on the natural history of osteosarcoma. Thus, pulmonary metastases have failed to develop in approximately 50%–90% of patients by 12–24 months. With maturity, the 5-year survival in most of the reported studies will probably plateau in the vicinity of 60%–70%. Therefore, it would appear more ethical and fruitful to administer potentially effective chemotherapy to all patients rather than to reconfirm (again) the bleak prognosis in concurrent, randomly assigned controls treated solely with surgery.

There are additional factors to support the use of chemotherapy. Many patients who relapse while receiving adjuvant chemotherapy demonstrate a reduced number of pulmonary metastases and/or a delay in their appearance (4). This has been a major element in facilitating retrieval of the relapsed patient by thoracotomy and other measures. This is clearly illustrated in patients treated at the M. D. Anderson Hospital and Tumor Institute (5). During this period, the medical attendants responsible for the treatment of osteosarcoma were relatively stable and the conceptual development and enthusiasm for retrieval of such patients accelerated only after the 1970s, when the new pattern of pulmonary metastases induced by chemotherapy emerged.

We endorse the suggestion that innovations in surgical technique may have been responsible for improved survival. However, many of these techniques were occasioned by chemotherapy. Thus, transmedullary amputation is utilized with increasing frequency, rotationplasty is performed in select cases, and limb salvage has been adopted with accelerating enthusiasm (6). Limb salvage is generally preceded by preoperative chemotherapy, which improves the safety of the procedure and reduces the possibility for local recurrence.

Finally, information on the influence of lag time and onset of symptoms, diagnosis, and survival is not available, and there is no evidence that physicians in the 1930s–1950s were reluctant to refer patients to major medical centers for immediate treatment once osteosarcoma had been diagnosed or suspected.

In summary, effective chemotherapy has improved the

cure rate, altered the pattern of pulmonary metastases, facilitated multidisciplinary intervention, and enhanced the opportunity for local resection with functional restoration. We are unshaken in our belief that historical controls constitute a valid basis of comparison, and any improvement in survival must be attributed principally to effective chemotherapy. As a result, the stated claim of our participation in a multi-institutional, prospectively controlled (surgery alone) randomized clinical trial is erroneous. We remain committed members of the Pediatric Oncology Group but have declined to participate.

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Reply: Role of Adjuvant Therapy in the Treatment of Osteosarcoma

The role of adjuvant therapy in the treatment of osteosarcoma is a controversial and emotionally charged subject of investigation. The areas of most intense debate are highlighted by Dr. Jaffe and his colleagues in their letter in response to a recent paper by Lange and Levine (1). The opposing views in this debate are presented in detail in the Lange paper. There is little question that the outlook for children with osteosarcoma prior to 1970 was dismal, and the 20% relapse-free survival and survival plateau were confirmed at several centers (2). There is also little doubt that the prognosis for these patients has improved significantly in the past 10 years, with 40%-60% of such patients now anticipated to be cured. How much of this improvement can be attributed to the use of adjuvant chemotherapy and how much to other, as yet undefined factors cannot be clearly deduced from the available data. Attributing the improved survival of children with osteosarcoma to the application of adjuvant chemotherapy presupposes that the historical experience for patients with osteosarcoma treated with surgery alone remains valid in the 1980s. Recent experience at the Mayo Clinic (3) has demonstrated an improved relapse-free survival experience for patients treated with surgery alone in the 1970s. Furthermore, in a randomized study from the Mayo Clinic, the group of children treated with surgery alone had a disease-free survival closer to 50% than the historical 20%. The Mayo Clinic experience is indeed based on a small series of patients, as noted by Jaffe et al, but this study remains the only randomized controlled trial addressing this issue.

The factors other than adjuvant therapy that could contribute to improved relapse-free survival for patients with osteosarcoma are discussed in detail by Lange and Levine. Jaffe et al are justifiably skeptical that the introduction of linear tomography is sufficient independently to account for improved relapse-free survival for patients with osteosarcoma diagnosed in the late 1970s (although the impact of newer generation CT scanners may be quite substantial). On the other hand, the combination of newer diagnostic techniques, heightened interest in the disease at major treatment centers, a better educated referral base, and more rapid and extensive patient evaluation and treatment might all contribute to improved relapse-free survival independently of adjuvant therapy. The use of historical controls in clinical trials is acceptable if there is assurance that factors other than therapy which might alter prognosis have not changed over time. Whatever factors are responsible for the change in relapse-free survival at the Mayo Clinic and elsewhere, numerous factors can be cited which make contemporary study patients different from historical controls. While the Mayo Clinic study remains to be confirmed, the data pose a direct challenge to the validity of the historical experience as a control for contemporary trials of adjuvant chemotherapy in osteosarcoma.

Since even the most favorable estimates predict a

40%–60% relapse-free survival rate for patients with osteosarcoma diagnosed in the 1970s–1980s treated with or without adjuvant therapy, reports of 90% relapse-free survival from the Memorial Sloan-Kettering Cancer Center (4) for patients treated with the T-7 and T-10 regimens are remarkable. These results, too, need to be confirmed in a multi-institutional setting. As discussed by Lange and Levine, it is difficult to understand these very favorable results reported by Rosen which have been achieved with chemotherapy regimens not significantly different from other regimens reporting only a 45%–60% relapse-free survival rate. In this regard, it is noteworthy that the projected results of the Sidney Farber Cancer Institute Protocol No. 3 have changed with further follow-up and the actuarial relapse-free survival rate at 5 years is 55% (A. Goorin, personal communication).

As additional evidence to support the use of adjuvant chemotherapy in osteosarcoma, Dr. Jaffe and colleagues cite the reduced number of pulmonary metastases and the delay in the appearance of these metastases in adjuvantly treated patients compared to historical controls. This has putatively facilitated treatment of relapsing patients. Here, comparison to historical patients is again confounded by the changes in supportive care, changes in treatment philosophy on the part of physicians, and improved diagnostic techniques. More frequent surveillance for metastases and use of the CT scanner have undoubtedly contributed to earlier detection of pulmonary nodules when they are limited in bulk, reduced in number, and more easily resected. Long-term salvage of patients after the development of metastases has been reported for patients who had been treated with or without the use of adjuvant chemotherapy (5).

Whether or not chemotherapy has facilitated advances in surgical management of osteosarcoma remains controversial. Transmedullary amputation has been recommended at many centers, and there is no evidence that such a procedure is associated with a higher local recurrence rate in patients not receiving adjuvant chemotherapy. The role for chemotherapy in improving the safety and local control rate of limb-sparing procedures which provide clean surgical margins likewise remains conjectural.

The controversies surrounding the management of osteosarcoma make it difficult to assess which components of current diagnostic and therapeutic measures have contributed to the improved survival of today's patients. If the disease-free survival of patients not treated with adjuvant chemotherapy exceeds 35%–40%, then the benefits of adjuvant chemotherapy (if any benefit can be demonstrated at all) are indeed marginal in view of the long- and short-term toxic effects of such therapy. The extent of the controversy surrounding this disease and the difficulties in comparing the group of children with osteosarcoma diagnosed in the 1980s with the historical patients have clouded interpretations of contemporary uncontrolled trials. Accordingly, investigators of the Pediatric Oncology Group have joined with those from the Dana-Farber Cancer Institute, the National Cancer Institute, The Children's Hospital of Philadelphia, and the London Solid Tumor Group in a controlled study of adjuvant chemotherapy in patients with nonmetastatic osteogenic sarcoma. As Dr. Jaffe indicates, patients from the M. D. Anderson Hospital are not being entered into the multi-institutional osteosarcoma study which is currently in progress. However, investigators from the Department of Pathology at the M. D. Anderson Hospital are participating as review pathologists for the study. It is hoped that this long overdue trial will clarify many of the controversies surrounding the management of osteosarcoma and provide an appropriate foundation for future studies.

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Leucovorin rescue has been used in at least four of the reported cases of vincristine overdose (1,2). It has already been suggested that the Food and Drug Administration urge the manufacturers of vincristine to recommend such treatment in the package insert (3). We report our observations on two patients with a similar histologic diagnosis, who received the same dose of vincristine on the same day in two neighbor institutions. One of these patients was treated with leucovorin rescue.

CASE REPORTS

Patient 1

A 42-year-old man underwent orchiectomy for a testicular teratocarcinoma and received chemotherapy for bulky abdominal disease. He was given two induction cycles, each consisting of two courses of vinblastine and bleomycin and two courses of doxorubicin and cisplatin, with the total dose of vinblastine being 45 mg/m^2. He was then considered to be in complete clinical remission. To cover a waiting period before the second-look laparotomy, 10 mg of vinblastine was to be given. Ten mg of vincristine (6 mg/m^2) was injected instead, due to a prescribing error. The patient was put under intensive care with parenteral fluids, furosemide (20 mg three times/day for 5 days), phenobarbital (400 mg/day for 4 days), and leucovorin (15 mg iv every 3 hours on the first day and every 6 hours for the following 4 days).

Vomiting was observed once, at 5 hours after the injection. Fever on the second day was easily controlled with phenacetin. Diarrhea on the first 3 days was replaced by constipation, and paralytic ileus was complete by Day 6. Treatment with ceruletide and bethanechol was tried without success. The ileus resolved gradually between Days 10 and 13. Mental confusion, agitation, insomnia, and hallucinations began on Day 3 and lasted 2 weeks. Areflexia, already present before the overdose, continued until the end of the sixth month. The patient reported myalgia and paresthesia of the hands and feet from Day 4 to Day 60, and moderate muscular weakness was observed during the same period. Glossitis was present during the second week. No signs of inadequate ADH secretion were observed. Wbc count nadir was 0.56×10^3 on Day 9 with 5 neutrophils/mm^3, recovering to 4×10^3 by Day 31. Platelet count nadir was 38×10^3/mm^3 on Day 5, recovering to 100×10^3 on Day 12. There was no anemia. Blood chemistry remained normal, except for the transaminases which were already slightly increased before and during the induction chemotherapy.

The neurologic examination remains normal 5 years after the overdose. Blood values are normal except for transaminases, which continue to fluctuate with the patient's alcohol intake.

Patient 2

A 28-year-old man with lung metastases of a testicular teratocarcinoma, appearing 4 months after orchiectomy and irradiation of abdominal lymph node metastases, received a first course of chemotherapy with vinblastine and bleomycin (total dose of vinblastine, 12 mg/m^2). A partial remission was documented when he was admitted for a second course after 3 weeks. Vinblastine (9 mg [6 mg/m^2]) and bleomycin were prescribed for the first day of treatment. Vincristine (9 mg) was injected instead of vinblastine. Treatment began on the second day of the overdose, with parenteral fluids and phenobarbital. No leucovorin was given.

Fever was observed during the first 3 days. Constipation began on Day 4. Intestinal paralysis was diagnosed on Day 7 and resolved on Day 10. There was no complete ileus; the patient passed stool every day under treatment with pyridostigmine. Mental confusion, agitation, insomnia, and hallucinations beginning on Day 4 were maximal on Day 6 and had disappeared by Day 12. Severe pain in the jaws, the face, and the neck were reported on Day 3 and lasted 10 days; however, the patient responded neither to routine analgesics nor to carbamazepine. Paresthesia and myalgia of the limbs appeared after 15 days and persisted for 3 weeks. Hyporeflexia, first seen in the sixth week, persisted for 2 months. Stomatitis was present on Days 5–9. Hyponatremia occurring during the second week could not be proven to be due to inadequate ADH secretion. Wbc count nadir was 2.6×10^3/mm^3 on Day 10 and platelet count nadir was 26×10^3/mm^3 on Day 3, with recovery on Days 12 and 9, respectively.

Blood values and physical examination were normal 4 months after the overdose. No later findings could be attributed to the vincristine overdose.

DISCUSSION

It is possible that the relatively major severity of leukopenia and neurotoxicity in the first patient was caused by the heavier previous treatment. However, a comparison of these two cases observed under similar conditions and of the known reports of vincristine overdose with and without leucovorin rescue (1,2) leads us to believe that, apart from animal experimentation, no reason exists today for recommending this procedure as a treatment for vincristine overdose.

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Cancer Research Supplement on Nutrition Available

Proceedings of "Nutrition in Cancer Causation and Prevention," a workshop conference, have been published in a supplement to the May 1983 issue of *Cancer Research*, the official publication of the American Association for Cancer Research. The workshop, sponsored by the American Cancer Society in Fort Lauderdale, Florida, October 18–20, 1982, was attended by 40 leading experts in nutrition and cancer research.

The supplement, "Workshop Conference on Nutrition in Cancer Causation and Prevention", summarizes the participants' work on the relationship between diet and cancer, and presents recommendations for future research strategies. The role of diet in the etiology of carcinogenesis is explored in papers on mutagens, nitrosamines, and polycyclic aromatic hydrocarbons in certain foods. The role of diet in the inhibition of carcinogenesis is highlighted in a section dealing with agents which act as suppressors of carcinogenesis, eg, selenium, retinoids, ascorbic acid, and plant sterols. Also included are articles on the association between dietary fat and cancer, as well as statistical studies on the incidence of cancer in certain geographic areas and among population groups with particular life styles. Finally, the difficulty of formulating public policy recommendations on nutrition is discussed. The American Cancer Society paid for the supplement's publication.

For copies of the supplement, contact: Cancer Research Editorial Office, Temple University School of Medicine, Fels Research Institute, W Bldg, Rm 301, Philadelphia, PA 19140.

The National Cancer Institute Clinical Investigator Award

Continuous application receipt dates: June 1, October 1, February 1

The National Cancer Institute announces the availability of Clinical Investigator Awards. The initiation of this award is intended to encourage recently-trained highly-qualified physicians to undertake careers in cancer research. The award is prompted by the chronic shortage of investigators particularly in surgical oncology, radiation oncology, preventive oncology, physiatry, nutrition, and epidemiology. It is expected to facilitate the awardees' transition to an independent basic or applied researcher. The award provides three years of support for successful candidates. Support is based upon a full-time, twelve-month staff appointment. Recipients of the award will receive salary support not to exceed $30,000 annually from NCI funds for the three-year period. Up to a total of $10,000 annually will be provided for supplies, equipment, travel, etc, which are deemed necessary for the research program.

To be eligible for an award, applicants must identify an established investigator to serve as a mentor. This sponsor must make a commitment to supervise and guide the candidate in his/her research. A well-detailed description of the research plan must be provided by the candidate in the application and evidence of institutional support must also be clearly demonstrated. Recipients must also agree to spend at least 75% of their time pursuing the research project proposed in the application for this training grant. Up to 25% of the grantee's time may be spent involved in teaching and/or other clinical activities relevant to their career development. Grants will be awarded to the recipient's parent institution and institutions will be allowed to apply for awards on behalf of individuals.

For more information, contact: Barney C. Lepovetsky, Chief, Cancer Training Branch, Division of Resources, Centers, and Community Activities, Blair Bldg, Rm 717, 8300 Colesville Rd, Silver Spring, MD 20910 (telephone 301-427-8898).

Twentieth National Reticuloendothelial Society Meeting

Portland, Oregon, October 9–12, 1983

The Scientific Program Chair for this meeting, to be held at the Portland Hilton Hotel, is Dr. Robert I. Mishell, University of California, Berkely, and the Local Host Chair is Dr. Jane M. Siegel, University of Oregon Health Science Center, Portland. The scientific sessions will consist of a workshop, three symposia, submitted papers, poster presentations, and a keynote address.

There will be two special award competitions held this year for submitted abstracts that will be

judged on the basis of their scientific merit and their presentation at the national meeting. The Presidential Awards will be for students and the Young Investigator Award will be for investigators under age 36. First prize for both competitions is a travel stipend to the International Congress of the Reticuloendothelial Society to be held at Mount Fuji, Japan, in 1984.

For more information, contact: Reticuloendothelial Society, c/o Dr. Sherwood M. Reichard, Medical College of Georgia, Augusta, GA 30912.

Seventh Annual Symposium on Advances in Cancer Treatment Research

Baltimore, Maryland, October 13–15, 1983

This timely symposium, to be held at the Hyatt Regency in Baltimore, features experienced and internationally recognized faculty, and is balanced between discussions of state-of-the-art cancer treatment and pending developments. Current research will be discussed in the context of possible therapeutic application. The symposium is designed to update physicians on recent developments in the area of cancer treatment by all modalities.

For further information, contact: Program of Continuing Education, University of Maryland School of Medicine, 10 S Pine St, Baltimore, MD 21201 (telephone: 301-528-3956).

American College of Physicians Medical Oncology Review Course

Honolulu, Hawaii, October 17–22, 1983

This popular review course, scheduled three weeks before the Medical Oncology Board exams, will be directed by Dr. Thomas C. Hall.

For more information, contact: Maxine Topping, Postgraduate Division, American College of Physicians, 4200 Pine St, Philadelphia, PA 19104 (telephone: .215-243-1200 or 800-523-1546).

Cancer Chemotherapy and Selective Drug Development

Brighton, England, October 24–28, 1983

This Tenth International Symposium on the biological characterization of human tumors is a multidisciplinary forum for the discussion of contemporary problems in cancer chemotherapy.

The plenary sessions include clinical topics: improved drug combinations, high-dose therapy, bone marrow transplantation, new drugs, drug resistance, treatment of specific cancer, immunotherapy, endocrine therapy, and control of infection; and preclinical topics: experimental models, drug resistance, pharmacokinetics, targeted chemotherapy, biological response modifiers, new drug development, and radio/chemosensitization.

The study/workshop groups cover topics in medicine, Control of Pain and Emesis in Cancer Patients; nursing, Bridging the Gap Between Science and Care; biochemistry, Chromatin as a Target in Cancer Chemotherapy; and hormone receptors, Hormone Receptors and Their Role in Directing Therapy.

Proffered papers and posters are invited on the themes of the symposium. Registration is restricted to 350 active participants.

For further information, contact: CCHTI Secretariat, Institute of Cancer Research, Block E, Clifton Ave, Belmont, Sutton, Surrey SM2 5PX, England.

Second European Conference on Clinical Oncology and Cancer Nursing

Amsterdam, the Netherlands, November 2–5, 1983

This conference, hosted by the Koningin Wilhelmina Fonds, Organisation for Cancer Control, the Netherlands, will be the first gathering of all European Scientific Oncology Societies at one time and will be held in the International Congrescentrum RAI.

The scientific program will consist of four parallel sessions on different subjects in the morning and afternoon where selected proffered papers will be communicated and discussed. Plenary sessions will follow and the parallel sessions and posters relevant to the particular subjects will be reviewed.

There will be opportunity at this integrated conference for nurses and doctors to meet and discuss problems in common. Future developments in oncology nursing and possible collaboration of European nurses will be discussed.

A social program for participants and a special excrusion program for accompanying persons have been composed.

For further information, contact: Second European Conference on Clinical Oncology, c/o Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, the Netherlands.

Current Concepts in Cancer Therapy, II

St. Louis, Missouri, November 3–5, 1983

This meeting will be presented by the Department of Surgery and the Office of Continuing Medical Education, Washington University School of Medicine in St. Louis. The program chairman is Marc K. Wallack, MD. The meeting will be accredited for 17 hours AMA Category 1, AAFP Prescribed, and AOA.

For further information, contact: Loretta Giacoletto, Office of CME, Washington University School of Medicine, Box 8063, 660 S Euclid, St. Louis, MO 63110 (telephone 314-454-3873).

New Perspectives in Human Lymphoma

Houston, Texas, November 9–12, 1983

This 27th annual clinical conference, held at the Shamrock Hilton Hotel, will focus on various controversial aspects of the diagnosis and management of lymphomas. Topics to be covered are pathogenesis and etiology, staging, and the use of various modalities of therapy, including, radiation, chemotherapy, immunotherapy, and bone marrow transplantation.

Speakers include: C. Berard, D. Bergsagel, C. Bloomfield, G. Bonadonna, R. Fisher, E. Frei, N. Gutensohn, M. Kadin, A. Levine, I. Magrath, L. Nadler, and L. Prosnitz. Conference co-chairpersons are Drs. Richard Ford, Lillian Fuller, and Fredrick B. Hagemeister.

For more information, contact: Office of Conference Services, Box 18, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave, Houston, TX 77030 (telephone: 713-792-2222).

Second International Conference on Human Tumor Markers

Vienna, Austria, February 20–22, 1984

This conference is sponsored by the International Society for Preventive Oncology (ISPO), the Austrian Cancer Society-Austrian Cancer League, and the Association of Clinical Scientists, USA. Sessions, structured as workshops, include an update on biological and clinical applications of tumor markers for risk assessment, cancer detection, and patient surveillance. Podium and poster presentations, panel discussions, and scientific and technical exhibits are scheduled. Registration includes round table luncheon discussions.

Abstracts of presentations on relevant topics are invited by *September 15, 1983*, which will appear in *Cancer Detection and Prevention* vol 7(1), 1984. An award for original communication of outstanding investigation(s) will be presented by ISPO.

For further information, contact: Human Tumor Markers Conference, AMEXPOB #790459, Dallas, TX 75379 (telephone: In US, 1-800-527-0297; Outside Europe, [usa-214]392-3663; In Europe, [austria 43-222]52-0544).

Fourth International Conference on the Adjuvant Therapy of Cancer

Tucson, Arizona, March 21–24, 1984

This conference will be held at the Tucson Convention Center under the sponsorship of the University of Arizona Cancer Center. Drs. Stephen E. Jones and Sydney E. Salmon will serve as conference co-chairmen. The deadline for submission of abstracts, prepared in the AACR/ASCO format, is *November 1, 1983.*

For further information, contact: Mary Humphrey, Conference Coordinator, University of Arizona Cancer Center, Tucson, AZ 85724 (telephone: 602-626-6044).

Twentieth Congress of the International Society of Hematology

Buenos Aires, Argentina, September 1–7, 1984

The scientific program for the congress will be centered on fundamental issues of hematology and will consist of plenary sessions, symposia, workshops, and poster discussion sessions. Included in the congress week will be a two-day educational program.

Spanish and English will be the official languages.

For further information, contact: Twentieth Congress of the International Society of Hematology, Viamonte, 2008, (1056) Buenos Aires, Argentina.

CORRECTION

In the article "Biochemical Pharmacology and Toxicology of Formycin Alone and in Combination With 2'-Deoxycoformycin (Pentostatin)" by Spremulli et al (*Cancer Treatment Reports*, Vol. 67, No. 3, March 1983, pages 267–274), the LD10 and LD50 for formycin + dCF were reversed in the printing of table 1. The three entries in the second line of table 1 should read (left to right): Formycin + dCF, 10, 19 (13.1–25.5).

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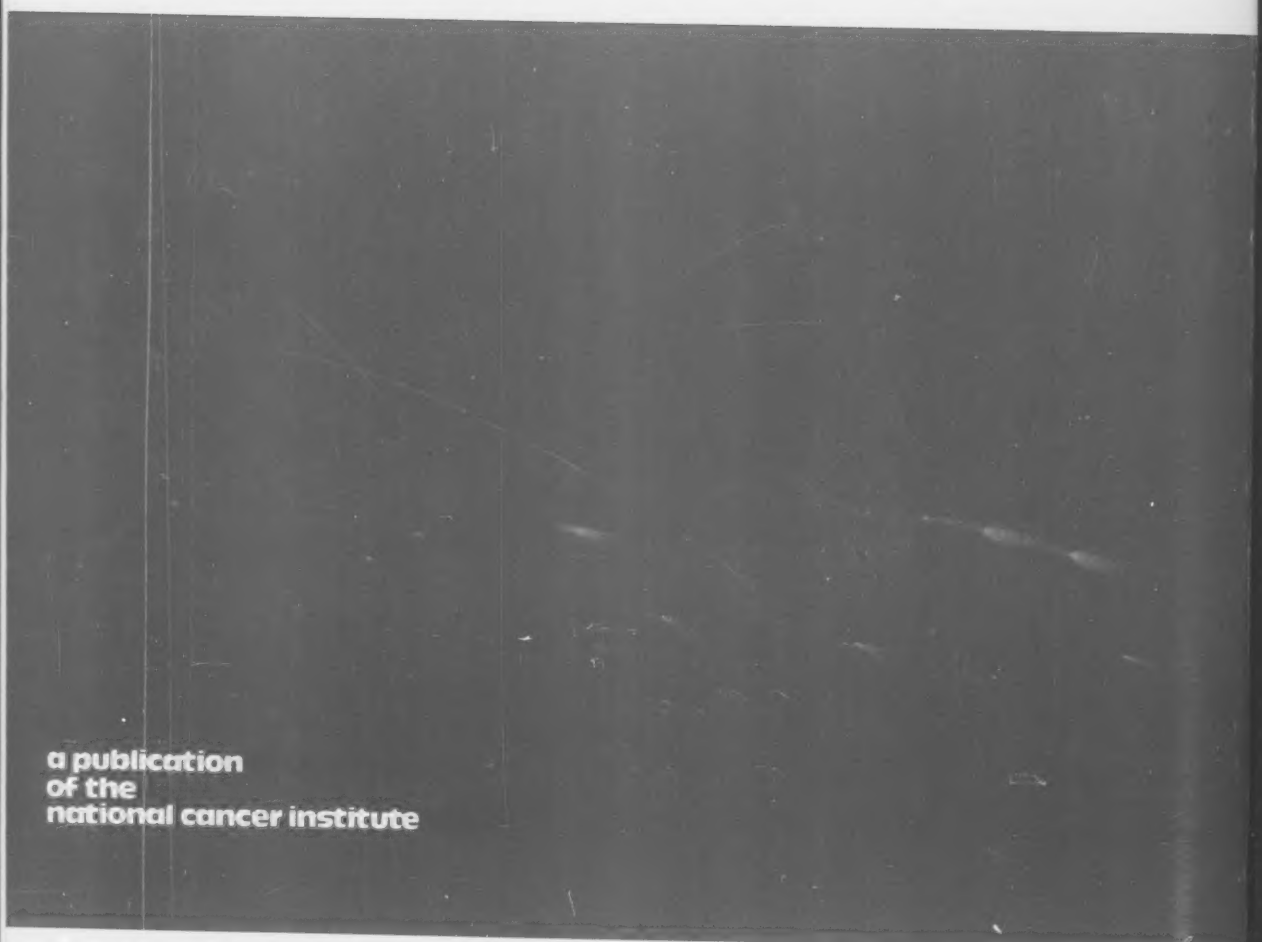

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